Identification and characterisation of a set of differentially expressed genes from *Glossina morsitans morsitans* refractory to a trypanosome infection

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

Nicholas Onyemauche Ejeh

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Declaration

I declare that this thesis and the work presented in it are my own work and that it has not been submitted in support of an application for another degree or qualification of this or any other University or other institute of learning.

Where other sources of information have been used, they have been appropriately acknowledged. The tsetse midgut 454 cDNA was prepared by Stella Lehane and was sequenced and assembled at The Centre for Genomic Research, University of Liverpool by Alistair Darby and Professor Neil Hall.

Dedication

This thesis is dedicated to my daughters Lucy, Onyi, Rosemary and my wife Chika for enduring a lot of hard times as a result of my studies and to the memory my late brother, Patrick Ejeh.

Abstract

Tsetse flies (Diptera: Glossinidae) are the vectors of African trypanosomiasis, a parasitic disease of humans and other animals that can be fatal if untreated. Trypanosomes undergo a complex life cycle in the fly involving a series of morphological and biochemical changes that culminate in the development of mammalian infective metacyclics in the salivary glands (*Trypanosoma brucei*) or the mouthparts (*T. congolense*). Once in the fly's midgut, the stumpy forms, which are thought to be pre-adapted for life in the tsetse midgut, differentiate into the procyclic form. However, the majority of flies can effectively get rid of parasites before they develop or establish an infection in the midgut. One would expect that as a result of the high disease burden in Africa, there would be a corresponding large number of flies that are also infected, yet paradoxically, just very few number of flies (<5%) can actually transmit the parasites to susceptible mammalian hosts. This means that tsetse flies are generally resistant to trypanosome infection, but the molecular mechanisms underlying this phenomenon are yet to be elucidated. The reasons for this natural refractoriness to a trypanosome infection are considered to be multifactorial and include the age of the fly, maturation of the fly immune system and peritrophic matrix among others, but so far few tsetse genes have been associated with this intrinsic phenotype.

In this thesis, I have identified and partially characterised several midgut genes from the tsetse vector, *Glossina morsitans*, that have been identified as differentially expressed (DE) in flies refractory to a trypanosome infection. A bioinformatics analysis of the 454 transcriptome data shows a total of 55 genes were DE in flies that were challenged with trypanosome. Four differentially regulated midgut genes (*i.e*. Chitinase (*CHIT*; GMOY000153), *O*-GlcNAc transferase (*OGT*; GMOY002400), secreted Phospholipase A_2 (*sPLA$_2$*; GMOY009713) and serine proteinase inhibitor (*SPI*; GMOY006016)) were further analysed *in silico* as representative of different metabolic pathways.

RNAi knockdown of midgut-expressed *CHIT*, *OGT*, *sPLA$_2$* and *SPI* genes significantly increased susceptibility to *T. brucei* infection in young files, suggesting that these genes are involved in the immune response to trypanosome infection in *G. m. morsitans*. However, reversion of the infection phenotype after RNAi silencing was not higher than ~40%, indicating that several proteins/pathways may be implicated in preventing establishment of a trypanosome infection in the tsetse midgut.

The potential role of the midgut-expressed $sPLA_2$ activity as a trypanocidal molecule was also studied. Over a period of two weeks, it was found that expression of midgut $sPLA_{2,}$ in flies receiving blood meals infected with bloodstream forms (BSF), is suppressed at the early stage of trypanosome infection, but its rises sharply after 14 days post-infection (dpi). The level of $sPLA_2$ expression in response to infection with procyclic forms (PCF) did not differ from the expression seen when flies were challenged with BSFs, ruling out the possibility of $sPLA_2$ expression being in response to the presence of mature PCFs. However, expression of $sPLA_2$ was augmented at 14 dpi in flies challenged with a higher parasite load, but not with either dead parasites or

bacteria, suggesting that its expression is somewhat dependent on the density of live parasite density. Surprisingly, when flies are fed with live *Staphylococcus aureus*, but not *Escherichia coli* there was a strong induction of $sPLA_2$ as early as 5 dpi, suggesting that its expression may be linked or modulated by the Toll pathway in response to certain pathogens. Furthermore, *in vitro* killing assays using recombinant $sPLA_2$ showed that procyclic trypanosomes were lysed after 24 hours when co-cultured with at least 50 μg/ml of active enzyme. Thus, it is hypothesised that at 14 dpi, when the trypanosome infection is well established in both the midgut and proventriculus of the fly, a release of PLA_2 may help control parasite infection, but is unable to clear it.

The data obtained from this thesis will offer new insights on the molecular dialogue that ensues between trypanosomes and tsetse following the ingestion of trypanosomes by tsetse in a blood meal.

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I would like to thank my wife Chika and my daughters, Lucy, Onyi and Rosemary for their love and support. I am particularly grateful to my parents, brothers and sisters for standing by me all these years and most especially my brother, Rev Fr. (Professor) Benedict Ndubueze Ejeh for bearing the financial burden of my studies and for making sure I was not left in the lurch during my academic programme.

Table of contents

List of Tables

List of Figures

List of Abbreviations

aa	Amino acid
AAT	African animal trypanosomiasis
AMP	Antimicrobial peptide
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
BSF	Bloodstream form
CSF	Cerebrospinal fluid
dNTP	Deoxy nucleoside triphosphate
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
Duox	Dual oxidase
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
NOS	Nitric oxide synthase
GFP	Green fluorescent protein
GlcNAc	*N*-acetyl-D-glucosamine
HAT	Human African trypanosomiasis
IMD	Immunodeficiency
iNOS	Inducible nitric oxide synthase
kDa	Kilodalton
LB	Luria-Bertani
LPS	Lipopolysaccharide
MIC	Minimal inhibitory concentration
mRNA	Messenger ribonucleic acid
NCBI	National Centre for Biotechnology Information
NO	Nitric oxide
NOS	Nitric oxide synthase
OD	Optical density
OGT	*O*-GlcNAc transferase
ORF	Open reading frame
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCF	Procyclic culture form
PCR	Polymerase chain reaction
PGRP	Peptidoglycan recognition protein
PLA_2	Phospholipase A_2
PM	Peritrophic matrix
PPO	Prophenoloxidase
PRR	Peptidoglycan recognition receptor
PTM	Post translational modification
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE	Standard error
SPI	Serine proteinase inhibitor
SRAA	Serum resistance associated antigen
STE	Sodium chloride-Tris-EDTA
TAE	Tris-acetate EDTA

Chapter 1.

Literature Review

1.1 General Introduction

African trypanosomiasis is a debilitating parasitic disease that affects both humans and animals. It is transmitted by the bite of a tsetse fly infected with trypanosomes (genus *Trypanosoma,* order *Kinetoplastida*) when taking a blood meal. The disease afflicts populations in rural sub-Saharan Africa, consisting mainly of the poor and less privileged that depend on fishing, hunting, agriculture or animal husbandry for their livelihood (Barrett et al., 2003). The human form of the disease, human African trypanosomiasis (HAT) also known as sleeping sickness is caused by *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*, two subspecies of *Trypanosoma brucei*. While *T. b. gambiense* which causes over 98% of reported cases occurs in central and west Africa and causes a more chronic infection that lasts for years with a latent period that takes months and sometimes years before symptoms become manifest, *T. b. rhodesiense* occurs in southern and east Africa and causes a more virulent and acute infection, which progresses more rapidly to the late stage involving the central nervous system (CNS) accounts for the remainder of cases (WHO, 2013a, WHO, 2014b).

Animal African trypanosomiasis, which is known locally as Nagana disease is caused by different trypanosome species with *T. vivax* and *T. congolense* being the major pathogens for livestock, especially cattle and other ruminants (Eyford et al., 2011, Rotureau and Van Den Abbeele, 2013). Other species that infect livestock include *T. simiae*, *T. godfreyi*, *T. b. brucei*, *T. uniforme*, and *T. suis* (Rotureau and Van Den Abbeele, 2013). African trypanosomiases have catastrophic socio-economic impact on the African continent. More than 8 million square kilometres of the Sub-Saharan African continent otherwise known as the “the tsetse belt”, an area of fertile land, are inhabited by tsetse flies, the vectors of African trypanosomiasis turning the area into a “green desert” because it is

deserted by humans and cattle resulting in huge economic loss (FAO, 2002). About 70 million people in 36 countries are at risk of contracting HAT because they live in the so called “tsetse belt” and are therefore exposed to tsetse bites (Simarro et al., 2012a). Sleeping sickness is usually fatal if left untreated and occurs in two stages – the haemolymphatic stage and the neurologic stage (Barrett et al., 2003). N’gana is responsible for high mortality rate in cattle leading to huge economic loss. Currently there is no vaccine and the drugs used for treatment of HAT cause serious side effects and sometimes toxic in addition to the fact that administration of the drugs involves a complicated posology and requires hospitalization of patients and trained personnel (Fevre et al., 2006, Brun et al., 2010). Also there is widespread increase in the incidence of drug resistance in the treatment of AAT (Delespaux et al., 2008b, Delespaux et al., 2008a).

Both male and female tsetse flies feed only on blood and as a result both can transmit trypanosomes to mammals during a blood meal. For a successful transmission of trypanosomes to a mammalian host, the parasites must undergo a complex cycle of development and maturation in the tsetse fly. Since tsetse flies are the exclusive cyclical vectors of trypanosomiasis, although mechanical transmission by other haematophagous insects such as tabanids and *Stomoxys* can occur in the case of *T. vivax* (Rotureau and Van Den Abbeele, 2013), and in light of the fact that all efforts to develop a vaccine for the disease have so far proved unsuccessful, vector-oriented control measures form the bedrock of the fight against African trypanosomiases. In light of the above, an in-depth knowledge of the molecular mechanisms of the crosstalk between trypanosomes and tsetse flies is of paramount importance and one that could lead to the unravelling of the transmission dynamics of these medically and economically important parasites.

1.2 Epidemiology of African trypanosomiasis

The geographical distribution of African trypanosomiasis is restricted to discrete areas or foci in sub-Saharan Africa where there are suitable habitats for the

tsetse fly vector, and there are areas where tsetse flies are found with no incidence of sleeping sickness (WHO, 2010). There are many species and subspecies of *Glossina* with varied environmental requirements leading to the focal nature of the distribution of the vector across the tsetse belt of Africa. Although both *T. b. gambiense* and *T. b. rhodesiense* cause HAT or sleeping sickness, the presentation, manifestation, duration and treatment regimens as well as the epidemiological patterns of the diseases caused by these subspecies are quite distinct and understanding these differences plays a major role in the formulation of control strategies for HAT (Welburn et al., 2001, Fevre et al., 2006). The Palpalis group of tsetse flies, especially *G. fuscipes* and *G. palpalis* are the principal vectors of *T. b. gambiense* while the *Morsitans* group are the vectors of *T. b. rhodesiense* (Welburn et al., 2001). The clinical presentation for both diseases consists of an early haemolymphatic stage and a late encephalitic stage involving the CNS. The clinical presentation is characterized by a variety of symptoms ranging from non-specific such as fever, headache, weakness and general malaise to a more specific organ dysfunction involving the heart, skin, liver, spleen, eye and the endocrine system (Atouguia JLM, 2000, Duggan and Hutchinson, 1966). The late stage occurs when the parasites invade the CNS and is insidious in onset and chronic in nature in *gambiense* disease but in *rhodesiense* disease it is acute with a duration of weeks or a few months (Kennedy, 2006). Progression from early to late stage of the disease is not a clear cut process with symptoms of both stages overlapping thereby making distinction between the two stages based on clinical presentation ambiguous (Atouguia JLM, 2000, Franco et al., 2014a). Because of the non-specific nature of the symptoms, HAT can be confused with other diseases with similar symptoms and may require continued re-evaluation of patients before a correct diagnosis can be made (Sahlas et al., 2002, Simarro et al., 2012b)).

1.2.1 Transmission cycle

For both forms of HAT, infection prevalence requires the involvement of the three factors that constitute the “epidemiological triangle” namely, human host, reservoir and tsetse fly, all co-existing in an environment that is a suitable habitat

for tsetse flies (Figure 1.1) (WHO, 2013a). The reservoirs of infection, be it human or animal can also be the host suffering from the disease depending on their behavioural interactions with the environment. The vectors (tsetse flies) are wholly dependent on a conducive environment for survival while the parasite is transmitted between hosts by the vector (Franco et al., 2014a). Human beings act as both host and reservoir for Gambiense HAT while for Rhodesiense HAT which is a zoonotic disease, the main reservoirs are animals. The role of animals as reservoirs of infection in Gambiense HAT is not clear although *T. b. gambiense* has been discovered in a variety of animals and are therefore regarded as potential reservoir hosts for this trypanosome species (Simo et al., 2006, Njiokou et al., 2006, Wastling et al., 2011, Nkinin et al., 2002, Njiokou et al., 2010, Cordon-Obras et al., 2009)). It has also been established that animals are susceptible to infection with *T. b. gambiense* when they are experimentally infected (Duke, 1931, Penchenier et al., 2005).

However it has been suggested that how often humans are exposed to the tsetse fly vector could be an important factor in the distribution of *T. b. gambiense* (Davis et al., 2011). Although the vectors for Rhodesiense HAT may be present but with the unavailability of infected reservoir, transmission cannot always occur (WHO, 2013a). Also the acute nature of the disease means that infected individuals are less likely to be present in the same environment harbouring tsetse flies as a result the transmission cycle of *T. b. rhodesiense* mainly involves the circulation of the parasites between non-human reservoirs and the vector with occasional transmission of the disease to humans (WHO, 2013a). In the case of Gambiense HAT, because an infected individual can still maintain normal activities during the long period of infection, the flies can transmit the disease between humans even though very few flies are infected with the parasites and this may be the reason why *gambiense* HAT accounts for most of the HAT cases (Baker, 1974).

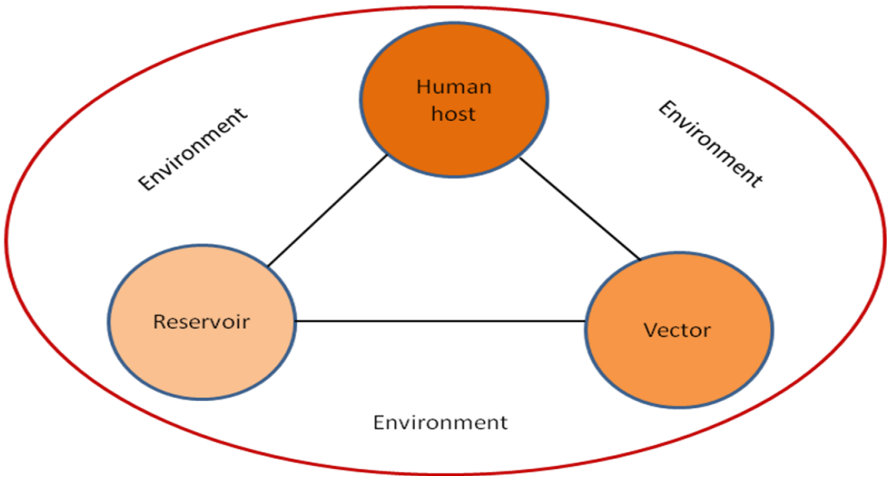

Figure 1.1 Epidemiological triangle for the transmission cycle of human African trypanosomiasis (adapted from WHO, 2013).

1.2.2 Risk factors

Combinations of various factors influence the rate of transmission of HAT. These include: increases in the likelihood of humans encountering tsetse flies, the site of contact between humans and tsetse flies, the intensity and frequency of contact and, in the case of *rhodesiense* HAT the existence of nonhuman reservoirs (Franco et al., 2014a).

Gambiense human African trypanosomiasis

The risk factors for Gambiense HAT is influenced by factors such as the type of environment harbouring the flies, the characteristics of the flies such as vector competence, behaviour, how often they come in contact with humans, life span and fly distribution and human activities within the different environments occupied by tsetse flies that are likely to bring humans in close proximity to tsetse flies (WHO, 2013a, Franco et al., 2014a). The risk for disease transmission increases when tsetse natural habitats are impinged upon by natural causes such as drought or human activities that destroy their habitats, for example, logging and farming (WHO, 2013a). About 31 species and subspecies of tsetse are known to inhabit various habitats ranging from forest for the *fusca*

group to savannah for the *morsitans* group and riverine and forest for the Palpalis group (Jordan, 1993).

The wide distribution of tsetse flies in humid forest habitats comes with an extensive range of hosts and human-fly contact does not occur on a regular basis because it is dependent on human activities that bring them in contact with tsetse flies such as hunting, fetching firewood, timber cutting for business, and clearing of forest for farming activities (Grebaut et al., 2001). Tsetse flies prefer to stay close to rivers and streams in the woodland savannah and riverine forest galleries. Humans who go to these water bodies to fetch water or carry out other domestic activities such as washing clothing or food, together with those engaged in mining and fishing are the ones mostly at risk of being bitten by infected tsetse flies (Robays et al., 2004, Kohagne et al., 2011, Moore et al., 1999). In-between forest and woodland savannah, the so called transitional vegetation where there are islands of vegetation which are frequently used for agricultural purposes also serve as suitable habitats for tsetse affording them the opportunity to sight hosts and this makes farming a risk factor for HAT in these areas (Moore et al., 1999).

Tsetse flies are also found in large populations in the mangrove areas where fishing and crustacean collection are associated with a high risk of transmission as well as cleared mangrove swamps used for rice cultivation. Pirogue jetties and encampments used for fishing provide an ideal environment for human-fly contact (Solano et al., 2009, Henry et al., 1982, Courtin et al., 2010). Areas where the original forest has been destroyed to plant cash crops such as cocoa, coffee, mango and banana form a suitable habitat for tsetse flies and people who work in these plantations are often exposed to tsetse bite and these areas therefore constitute transmission sites for plantation workers (Meda et al., 1993).

In as much as Gambiense HAT is regarded as a rural disease, there have been occasional incidences of the disease in urban areas where transmission has been associated with seasonal travels to neighbouring villages for cultivating fields and agricultural activities or in suburban outskirts of cities closer to transitional vegetation areas and therefore serve as a suitable habitat for tsetse,

but with a small number of alternative hosts (Fournet et al., 1999, Courtin et al., 2005, Robays et al., 2004). Also the disease is known to affect mainly young adults because they are the ones engaged in productive activities that predispose them to tsetse bite (Pepin and Meda, 2001, Paquet et al., 1995, Moore et al., 1999). Because children are less exposed to flies during the day, the rate of infection in children is low compared to adults except in some areas where children are engaged in at-risk activities such as fishing and leisure activities in water areas (Vanhecke et al., 2010, Abel et al., 2004).

Infection prevalence is not gender-specific but the behaviours and activities in certain areas determine which sex group is most at risk of contracting the disease. For instance, in areas where mining, hunting, or fishing constitute at-risk activities, infection prevalence is higher in males than in females, whereas in areas where the risk of infection is associated with cultivation and domestic activities at water points such as transitional vegetation, similar infection prevalence are found among males and females (Pepin and Meda, 2001, Paquet et al., 1995, Moore et al., 1999, Abel et al., 2004). Short-term travellers from nonendemic areas are less exposed to the vectors because tourists rarely visit the rural areas in which Gambiense HAT is transmitted. Immigrants and expatriates who have lived in endemic areas for extended periods are usually the ones diagnosed with occasional Gambiense HAT (Migchelsen et al., 2011, Simarro et al., 2012b).

It has been observed that more than one member of a given family may be infected with disease at the same time and the risk of a child having Gambiense HAT increases significantly if the mother had had the disease and siblings are more likely to contract the disease if one of them is infected (Pepin and Meda, 2001). This clustering of cases or familial aggregation could be attributed to genetic susceptibility or similar, but not necessarily simultaneous exposure to the vector and shared behavioural risk factors (Pepin and Meda, 2001). It has however been suggested that the later might be the case rather than the former (Khonde et al., 1997, Henry, 1981).

Rhodesiense human African trypanosomiasis

There are two different settings for the transmission of Rhodesiense HAT and therefore different situations for contracting the disease (Franco et al., 2014a). Wildlife and livestock are the main reservoirs for infection. In areas where wildlife constitutes the main reservoir, the more active and working-age groups are the ones at highest risk of being infected because they are the ones engaged in activities associated with entry into tsetse habitats such as national parks and game reserves (Franco et al., 2014a). The people most likely to be infected are the ones living very close to the wildlife preserved areas who engage in occupational activities and exploitation of national resources such as hunting and poaching, fishing, honey and firewood collection.

This is also the case for tourists who go to national parks for sightseeing as well rangers and park wardens who look after these parks (Kinung'hi et al., 2006, WHO, 2013a, Simarro et al., 2012b, Pepin and Meda, 2001, Zoller et al., 2008). Livestock may also serve as the main reservoir for Rhodesiense HAT in which case activities linked to cattle raising increases the risk of infection and those living around are also exposed (Franco et al., 2014a). Familial aggregation in Rhodesiense HAT is mostly attributed to common exposure to vectors (Zoller et al., 2008, Okia et al., 1994). The ecology of *Glossina* is such that the population densities of flies reaches a peak after the rainy season and there is a link between the density of *Glossina* and the transmission of Rhodesiense HAT with peaks of transmission occurring in the warmer months after the rainy season (Smith et al., 1998).

1.2.3 Incidence of disease and trends in numbers of reported cases

There is optimism that there is a possibility of eliminating sleeping sickness as a result of the decline in the number of cases after a period characterised by severe epidemics (Welburn et al., 2009). Various factors have been linked to the epidemics of sleeping sickness in Africa; they include increased movements and displacement of populations and modifications to the environment (for example, clearing of forests and the use of intensive agriculture to generate cash crops) as well as conflict and socio-political instability which go hand in hand with

persistence and resurgence of infection (Berrang Ford, 2007, WHO, 2013a). There have been some major epidemics of the disease since it was discovered but since 2000, there have been a sustained decrease in the number of notified cases with the figure falling to below 10,000 new cases in 2010 (figure 1.2) as a result of the reinforcement of control programmes by WHO in partnership with some international organisations and national control programmes of affected countries (Franco et al., 2014b) as well as a better understanding of the epidemiology of the disease (Simarro et al., 2010).

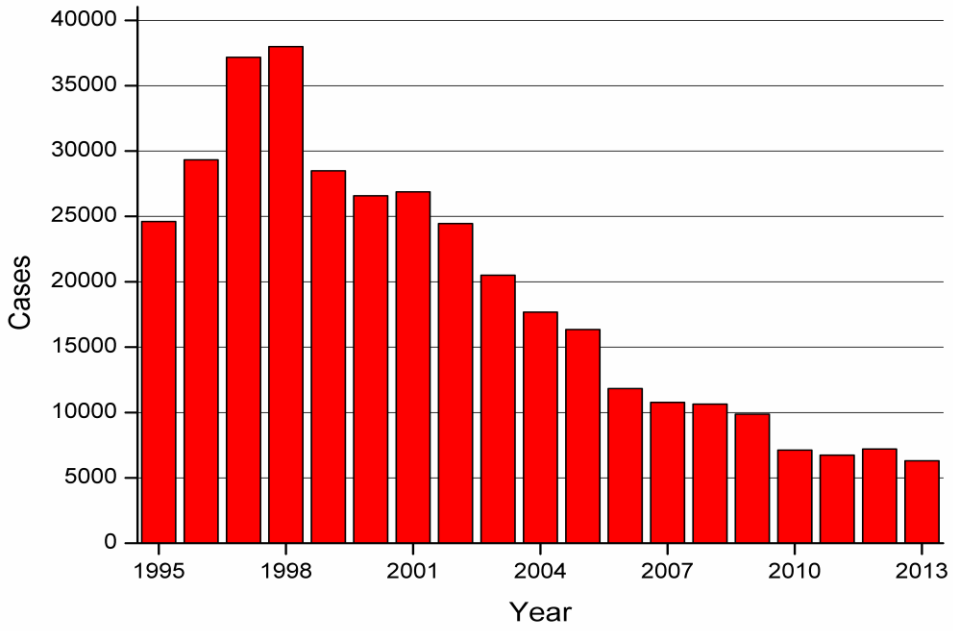

Figure 1.2 Total number of new cases of HAT (1995-2013). Cases reported to the World Health Organisation by disease endemic countries. Data from the World Health Organisation (WHO, 2013a, Simarro et al., 2013, WHO, 2013b, Franco et al., 2014a)

1.2.4 Epidemics of Gambiense human African trypanosomiasis

The origin of Gambiense HAT epidemics have been linked to various factors chief among which include socio-political changes that have led to sudden changes in the environment, displacement of populations as a result of war or civil strife, changes in human practices, and inadequate or lack of effective

control programmes in affected countries where the health care system is weak (Ekwanzala et al., 1996, Stanghellini and Josenando, 2001, Moore and Richer, 2001). Presently there is a decrease in the number reported cases of *gambiense* HAT of about 76% since 2000 (Franco et al., 2014a) with about 7000 reported new cases since 2011 (WHO, 2013a, Franco et al., 2014b, Simarro et al., 2013). In as much as this is encouraging, the figure should be regarded with caution because the disease is known to affect the poor who live in rural communities with little or no access to health infrastructure, and cases may therefore be unrecognised or unreported; also some endemic areas are difficult to access either because of security problems, challenging topography or extreme remoteness of these areas (WHO, 2013a, Franco et al., 2014b, Simarro et al., 2011, Simarro et al., 2013, Mumba et al., 2011, Chappuis et al., 2010). In the light of the above problems the number of reported cases if far below the number of actual cases and is estimated currently to be about three times the present figure (WHO, 2013a, Franco et al., 2014b, Simarro et al., 2011).

1.2.5 Epidemics of Rhodesiense human African trypanosomiasis

Rhodesiense HAT can occur in both endemic and epidemic forms depending on the main reservoir that is found in a given area (Franco et al., 2014a). In areas where both wildlife and livestock are the reservoirs of infection the disease is prevalent in its endemic state with stable, low incidence (WHO, 2013a). In areas where wildlife constitute the main reservoir, human cases occur occasionally although there may be long periods of years when there are no cases (WHO, 2013a, Allsopp, 1972, Simarro et al., 2011, Kaare et al., 2007). In areas with livestock as the main reservoir, there are more opportunities for interaction between humans, livestock, and vector and as a result cases usually occur more regularly (Odiit et al., 2006, Abaru, 1985). In both cases epidemics can occur as a result of civil strife and other major social and environmental disruptions that force people to move with their livestock to areas infested with tsetse flies thereby increasing human-tsetse fly contact which may result in more people being infected (Hide et al., 1996, Jelinek et al., 2002, Kaare et al., 2007, Morris, 1959, Berrang Ford, 2007, Selby et al., 2013).

The movement of domestic animals for economic purposes may result in the extension of an existing foci of infection or its introduction into a new area (Welburn et al., 2001). Like Gambiense HAT, the number of reported cases from endemic countries has declined since 2000 (WHO, 2013a, Simarro et al., 2011, Simarro et al., 2013). This trend is particularly true in areas that have witnessed an extensive modification of the physical environment in the form increase in population density which has led to the expansion of farmed areas and reduction of habitats for wildlife (WHO, 2013a, Franco et al., 2014a). Both forms of HAT occur in Uganda as a result of movement of infected livestock (Picozzi et al., 2005). Movement of livestock across the region will mix animals from regions endemic for both forms of HAT and a potential long-range spread of the parasites in years to come with a consequent major impact on diagnosis and treatment of the disease (WHO, 2013a, Picozzi et al., 2005)

1.2.6 Geographical distribution

The geographical distribution of both Gambiense and Rhodesiense HAT is wide but discontinuous, the disease being limited by the patchy distribution of the tsetse belt across Africa beyond which the disease does not occur (Welburn and Maudlin, 2012) (Figure 1.3). These are areas where the environment supports all the three elements in the transmission cycle – the host, the reservoir, and the vector and transmission can therefore take place (Franco et al., 2014a). The distribution of the disease is however more discrete than its vector species because there are areas where tsetse flies are found but HAT is not prevalent in contrast to the distribution of AAT which is found right across the tsetse belt (Welburn and Maudlin, 2012). Environmental changes brought about by human or livestock movements can cause alterations in transmission intensity, but foci of transmission remains more or less stable over time (Franco et al., 2014a, Paquet et al., 1995).

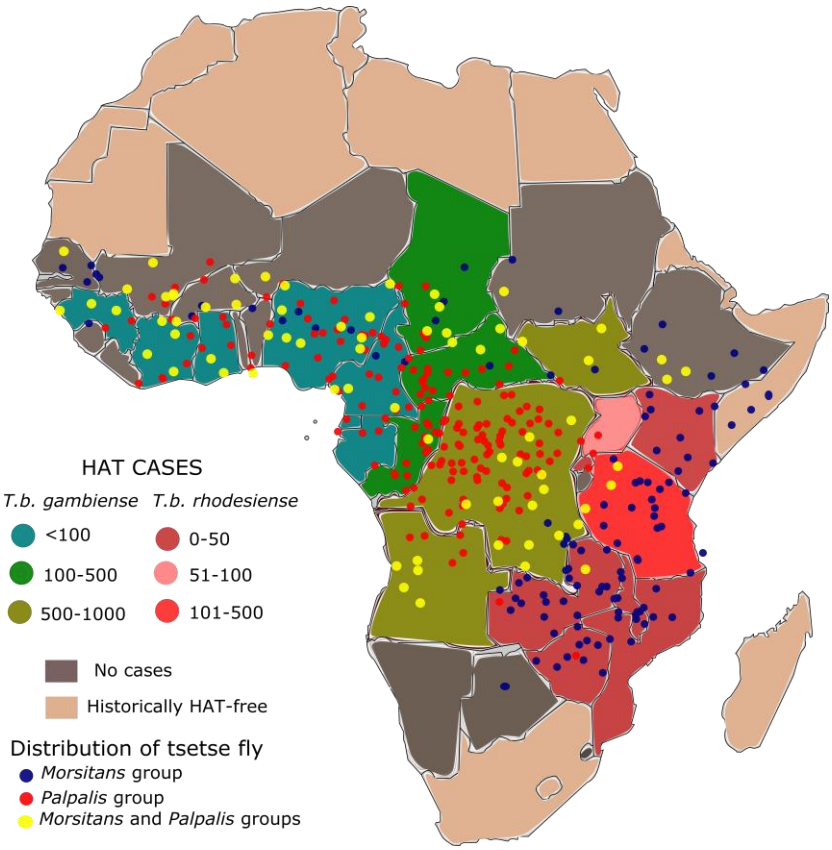

Figure 1.3 Map showing cases of Gambiense and Rhodesiense sleeping sickness (2000-2009) and distribution of tsetse fly in sub-Saharan Africa (Adapted from (Simarro et al., 2010)).

There are currently around 360 HAT foci in 36 sub-Saharan African countries with 300 Gambiense HAT foci in 24 countries of Western and Central Africa and 60 Rhodesiense HAT foci in 13 countries of Eastern and Southern Africa (Uganda having both forms), with most of these foci being in rural and remote areas (Cecchi et al., 2009, WHO, 1998, WHO, 1986, WHO, 2014a, WHO, 2013b). For Gambiense HAT, these foci are classified according to the intensity of disease transmission from very high levels to moderate to very low levels of transmission (Simarro et al., 2013, Simarro et al., 2012a), while for Rhodesiense HAT, they are classified according to the pattern of transmission depending on whether wild animals or cattle are the reservoir of infection (Simarro et al., 2013). In some areas however, both scenarios are prevalent leading to a mixed

transmission pattern with the reservoir being either wild animals or cattle (Franco et al., 2014a).

1.3 Sleeping sickness

T. b. gambiense and *T. b. rhodesiense* HAT occur in two sequential stages, the haemo-lymphatic stage occurs first followed by the second or meningo-encephalitic stage which occurs when the parasites invade the CNS; however most of the symptoms of the two stages can overlap without a clear distinction between them (WHO, 2013a). Both forms of disease differ in many ways including clinical presentation. Whereas *T. b. gambiense* HAT is a chronic and slow-evolving disease with an average duration of about 3 years, with the first stage lasting almost as long as the second stage, *T. b. rhodesiense* HAT is usually an acute febrile illness which progresses rapidly to death within weeks or months (Checchi et al., 2008, Odiit et al., 1997). However, variations in clinical presentations do occur most likely due to a combination of parasite and host genetic factors.

1.3.1 *T. b. gambiense* infection

The average duration of *T. b. gambiense* infection is approximately 3 years (Checchi et al., 2008). In some cases patients can recover from first stage infections without treatment (Jamonneau et al., 2012). Staging of disease relies heavily on the outcome of CSF examination because, although some signs and symptoms can be linked with either the first stage (e.g. high fever) or the second stage (e.g. neuropsychiatric disorders), none is considered characteristic for each stage (Burri et al., 2014) . It is difficult to ascertain the incubation period of *T. b. gambiense* infection since most infections occur in residents who must have been bitten multiple times by tsetse flies, sometimes on a daily basis and as a result the exact time of infection is unknown (Urech et al., 2011, Burri et al., 2014). However, the interval between infection and the manifestation of systemic symptoms lasts between weeks or months and the appearance of chancre (if

present) can occur in a matter of days following tsetse bite and disappears spontaneously in 1-2 weeks (Burri et al., 2014).

Systemic signs and symptoms are more pronounced and occur more frequently during the first stage of the disease than the second stage (Blum et al., 2006). Fever is irregular with episodes lasting 24 hours to several days and coincides with waves of parasitaemia marking the invasion of the host by the parasites and the onset of immune response (Blum et al., 2006). Pruritus occurs in both stages but increases in frequency with the duration and severity of the disease and therefore more frequent during the second stage (Blum et al., 2006). Local or generalised enlargement of the lymph node is typical most especially the posterior cervical lymph nodes (Winterbottom's sign), but can be found elsewhere (Burri et al., 2014).

Headaches and mood or behavioural changes are nonspecific neurological or psychiatric symptoms that are commonly observed in both stages of the disease but are seen more persistently and with more intensity as the illness progresses (Blum et al., 2006).

Sleep disturbance is a typical symptom in the second stage though some first-stage patients complain of nocturnal insomnia or daytime sleep but these symptoms are mostly associated with second-stage patients (Blum et al., 2006). Mental disorders are common and can be observed during the first stage of the disease and may be confused with primary psychiatric illness leading to wrong diagnosis (Sahlas et al., 2002, Urech et al., 2011). Most neuropsychiatric disturbances are not permanent since they can be reversed by administering anti-trypanosomal drugs (Buguet et al., 2005). However, if treatment was administered when the disease was at an advanced stage, irreversible sequelae of various degrees such as delayed sexual maturity or decreased academic performance (observed in children) may occur (Aroke et al., 1998).

1.3.2 *T. b. rhodesiense* infection

Unlike in *T. b. gambiense* infection, a broader spectrum of clinical presentation is often seen in *T. b. rhodesiense* infection and this may vary from a chronic disease pattern to the classical acute form, which may even differ between

different areas within the same country (Songa et al., 1991, MacLean et al., 2010). Like in *T. b. gambiense* infection, the incubation period for *T. b. rhodesiense* disease is difficult to ascertain, but has been estimated to last weeks in areas with acute clinical presentation and months in areas with a more chronic clinical presentation (Burri et al., 2014). Trypanosomal chancre at the site infective bite is seen more frequently than in *T. b. gambiense* infection (MacLean et al., 2010, Boatin et al., 1986, Buyst, 1977, Wellde et al., 1989, Urech et al., 2011). There are differences in *rhodesiense* HAT disease profile between distant foci and even those within close proximity with fever and headache being the main symptoms in the early stage of the disease in some foci whereas tremor and sleepiness were the main symptoms in others (MacLean et al., 2010).

Unlike in *gambiense* HAT, enlarged lymph nodes are mainly found in submandibular, axillary and inguinal regions rather than posterior cervical, and oedema of the face and legs are also observed more frequently in *rhodesiense* than in *gambiense* form of the disease (Kuepfer et al., 2011, Boatin et al., 1986). Cardiac involvement is a common feature of *rhodesiense* HAT which may lead to cardiac failure and finally death (Lejon and Buscher, 2005). Neurological signs and symptoms which are mainly seen in second-stage of HAT are similar to those found in *T. b. gambiense* infections, however, the progression towards coma and death occurs faster in *T. b. rhodesiense* infections (Burri et al., 2014).

1.4 Diagnosis

Clinical signs and symptoms of tsetse-transmitted trypanosomiasis cannot be taken as being specific for the disease; neither does it justify treatment considering the relative toxicity of all drugs currently in use. Demonstration of the parasite therefore is of utmost importance (Burri et al., 2014). Invariably, diagnosis relies on the employment of direct techniques that confirm the presence of trypanosomes in body fluids by microscopic examination or indirect serological methods or by molecular techniques (OIE, 2014). The diagnosis of *T. b. gambiense* HAT involves initial screening of suspected cases followed by diagnostic confirmation and finally, if positive, staging (Chappuis et al., 2005).

For *T. b. rhodesiense* HAT, screening relies on clinical signs and symptoms and diagnosis is usually by direct examination of blood while staging is done in the same way as for *T. b. gambiense* (Jelinek et al., 2002, Chappuis et al., 2005).

1.4.1 Antibody detection test

The card agglutination test for trypanosomiasis (CATT) is the primary screening tool for the diagnosis *T. b. gambiense* infections. It is a cheap, rapid and easy to use serological test that is used for the detection of specific antibodies in individuals infected with *T. b. gambiense* (Magnus et al., 1978). The test can also be performed on plasma or serum dilutions to improve specificity and mostly used to reduce the number of false-positives prior to parasite detection. However, there is no consensus as to the cut-off to be used as the threshold for interpreting whether or not the reaction is positive and for this reason parasitological test remains the gold standard and should be performed as a confirmatory test (WHO, 2013a). At present, there is no similar test for *T. b. rhodesiense* and field screening still relies on clinical signs and symptoms, but parasite detection in the blood is relatively easier for this form of the disease because trypanosomes are numerous in the blood and diagnosis is therefore mostly by examination of stained thin or thick blood film (Jelinek et al., 2002, Chappuis et al., 2005, Duggan and Hutchinson, 1966). Alternative serological tests, such as enzyme-linked immunosorbent assay (ELISA), immunofluorescence assays, and LATEX/*T. b. gambiense* test are also available but are not used for routine field diagnosis.

1.4.2 Trypanosome detection

Parasite detection is very important in the proper diagnosis of African trypanosomiasis (Burri et al., 2014). Body fluids such as blood, lymph node aspirate and CSF are examined microscopically for the presence of trypanosomes and thus provides direct evidence for trypanosome infection (Chappuis et al., 2005). The parasites may also be detected in bone marrow aspirates or ascites fluid. Failure to demonstrate parasites does not necessarily

mean lack of infection since parasite numbers can be quite low and below detection limit especially in *T. b. gambiense* infection (Chappuis et al., 2005). For this reason it is recommended that methods that allow for the examination of larger sample volumes such as concentration techniques be used for the diagnosis of *T. b. gambiense* infections to increase the chances of parasite detection (WHO, 2013a). To maximize the chances of parasite detection, it is essential to keep the time between sample collection and examination as short as possible to avoid immobilisation and lysis of trypanosomes, and if the samples are not to be examined immediately after collection (> 1 h), it should be stored at 4-8 ˚C (WHO, 2013a).

Blood films (thin, thick or wet) are used for direct detection of trypanosomes. While wet blood films are used for detection of motile trypanosomes, thick and thin blood films stained with Giemsa are used for detection of fixed trypanosomes. Wet and thin blood films have low sensitivity but examination of thick blood film slightly improves sensitivity since a larger volume of blood (about 20 µl) is examined (Chappuis et al., 2005). Aspirates from chancres or lymph nodes are a good source of parasites and can be examined as a wet preparation or fixed and Giemsa-stained preparation (Chappuis et al., 2005).

1.4.3 Molecular methods

A wide range of molecular tests are available; however, none have been validated for routine diagnostic purposes (Chappuis et al., 2005, Deborggraeve and Buscher, 2010). The current molecular diagnostic methods are not yet suitable for use at the primary-care level because of lack of the requisite infrastructure in the rural areas where the disease is endemic and as a result, use of polymerase chain reaction (PCR) cannot be used for first-line diagnosis of HAT in endemic areas (Deborggraeve and Buscher, 2010). Moreover, a positive PCR does not necessarily mean the presence of live trypanosomes, and this makes it difficult to accurately assess treatment outcome (Deborggraeve et al., 2011). Also most of the current molecular techniques are not able to exclude transient infection with non-pathogenic species of trypanosomes such as *T. b. brucei* (Deborggraeve et al., 2008).

Newer and faster techniques such as the human African trypanosomiasis-PCR-oligochromatography (HAT-PCR-OC), (Deborggraeve et al., 2006) and the Loop-mediated isothermal amplification (LAMP) (Riehle et al., 2007) are also used. For now molecular diagnostic techniques are yet to meet the requirements needed for them to be used in primary healthcare facilities and as a result diagnosis of sleeping sickness in Africa still relies on conventional methods such as antibody detection in an agglutination test and/or parasite detection by direct microscopy (Deborggraeve and Buscher, 2012).

1.4.4 Determination of the stage of the disease

Following the confirmation of infection, it is vital that the stage of the disease be accurately ascertained in order to administer the appropriate treatment to avoid unnecessary toxic side effects of the drugs used for treatment (Bouteille and Buguet, 2012). For the fact that majority of deaths can be traced to treatment-related acute encephalopathies (Pepin et al., 1994), it is important that tests be carried out to ascertain the stage of the disease before treatment can begin. Staging is done by examining CSF obtained by lumber puncture to look for the presence of trypanosomes and/or a raised white blood cell count (>5 cells/µl) (Organization, 1998). The sleep-wake cycle could be another useful marker for second-stage disease. The sleep-wake patterns of second-stage patients are completely disrupted but the disruption were ameliorated after treatment for second-stage HAT was administered. However, similar alterations may be observed in some patients with first- or “intermediate”-stage infection and as a result, more work is needed to assess the relationship between the second-stage HAT and disruption of sleep structure (Buguet et al., 2005).

1.5 The parasite

Understanding the biology of African trypanosomes is vital because it will enable us understand the biological attributes of these organisms that differentiate them from that of their mammalian hosts or vectors. These features that distinguish trypanosomes from their mammalian hosts and/or vectors must be understood

since they represent potential drug targets that may be exploited to meet the goal of elimination of HAT.

1.5.1 Classification of African trypanosomes

The African trypanosomes belong to an evolutionarily ancient protozoan order known as the kinetoplastida and genus *Trypanosoma* (Barrett et al., 2003). Based on the mode of transmission by their insect vector, the genus *Trypanosoma* is divided into the stercorarians and salivarians (Hoare, 1966, Levine et al., 1980) (Figure 1.4). The stercorarians develop in the posterior gut of their vector and infection of the vertebrate host occurs via faeces while the salivarians develop in the anterior gut of their invertebrate vector and later migrate towards the salivary glands (*T. brucei*) or the mouthparts (*T. congolense*) where the infective forms for vertebrates develop and infection of the mammalian host occurs via saliva (Baral, 2010).

All pathogenic African trypanosomes belong to the salivarians because they are injected into mammalian blood via saliva when an infected tsetse fly takes a blood meal (Rotureau and Van Den Abbeele, 2013). The *T. brucei* group which has three subgenera (*T. b. brucei, T. b. gambiense* and *T. b. rhodesiense*) infect cattle and humans: while *T. b. brucei* causes N'gana, a cattle disease in Africa, *T. b. gambiense* and *T. b. rhodesiense* which are morphologically indistinguishable, infect humans causing sleeping sickness in West and Central Africa and East and Southern Africa respectively (Baral, 2010). While *T. equiperdum* causes dourine, a venereal disease in horses and related animals and *T. evansi* causes surra in vertebrate animals, *T. congolense* and *T. vivax* are however, the major causes of animal African trypanosomiasis (Baral, 2010).

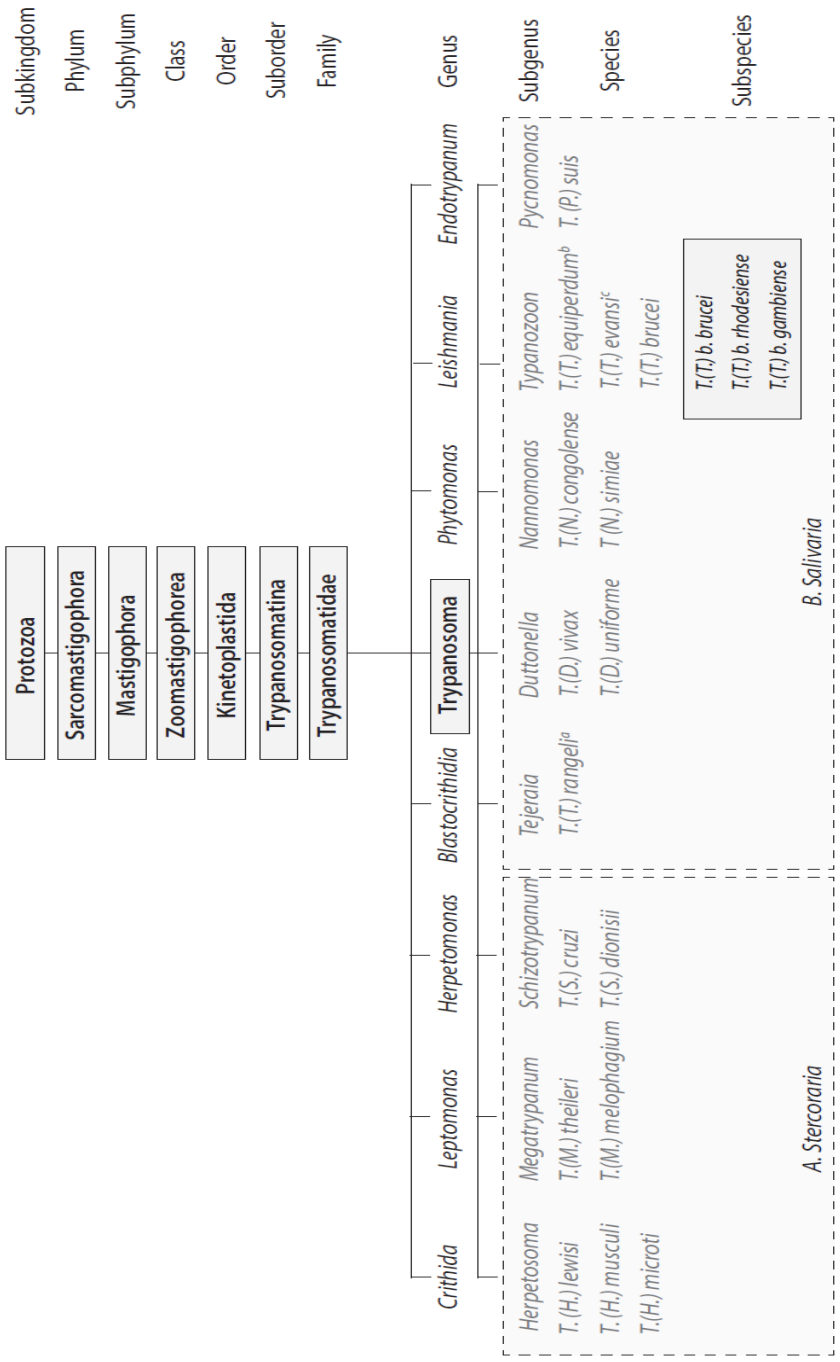

Figure 1.4 Classification of mammalian trypanosomes (Levine et al., 1980).

1.5.2 Morphology

African trypanosomes are unicellular, flagellated protozoan parasites that live extracellularly in the bloodstream. They are also found in other body fluids such

as lymph and cerebrospinal fluid (CSF). Morphologically, African trypanosomes are spindle shaped measuring about 20 µm by 5 µm with two principal nucleic acid-containing organelles, the nucleus and the kinetoplast and a single flagellum which originates at the posterior part of the body and exits the cell through the flagellar pocket (FP) (Figure 1.5). The flagellum runs along the cell body towards the anterior pole and is attached to the cell body by the flagellum attachment zone (FAZ) (Field and Carrington, 2009). Trypanosomes spend most part of their life cycle as two distinct forms- trypomastigotes and epimastigotes (Leung et al., 2014).

The relative positions of the nucleus, kinetoplast and the exit point of the flagellum along the anterior-posterior axis differ between these morphological stages. In trypomastigotes the flagellum emerges near the posterior end while in epimastigotes the position of emergence of the flagellum is at the centre of the cell (Leung et al., 2014) (Figure 1.6). The kinetoplast is the parasite's mitochondrial genome which is said to be larger than the mitochondrial genomes in other eukaryotic cells (WHO, 2013a). The flagellum apart from its involvement in the motility of the parasites is considered to play a role in microfluidic constant sweeping of the trypanosome cell surface and this is considered to be important particularly in the removal of surface bound antibodies leading to endocytosis and subsequent destruction (Engstler et al., 2007).

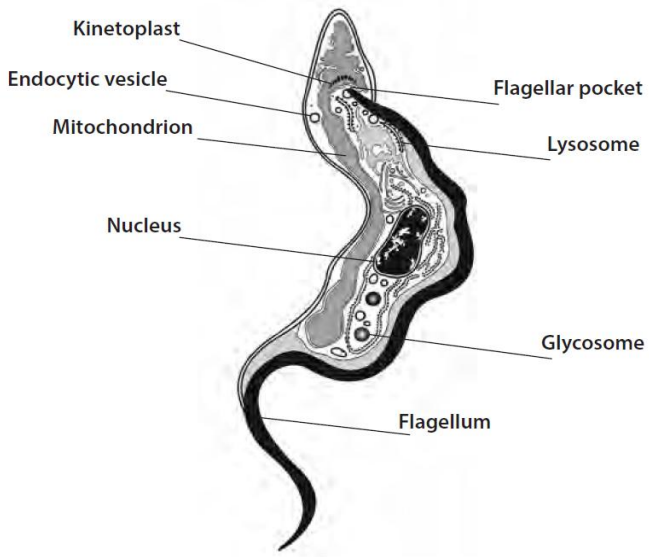

Figure 1.5 Structure of blood form African trypanosome showing the main intracellular features (WHO, 2013).

1.5.3 Life cycle of African trypanosomes

African trypanosomes have a complex life cycle which alternates between mammalian hosts and arthropod vectors. These trypanosomes must undergo cyclical development in the tsetse fly before they can infect a vertebrate. After completion of the parasitic cycle in the vector, it becomes infective and the fly can transmit the parasites to a vertebrate host when taking a blood meal and remains infective for life. For this reason African trypanosomes are said to be cyclically transmitted by tsetse flies. The life cycle involves different stages with distinct morphological forms and biochemical physiology (Figure 1.7). The flies become infected after taking up the parasites during a bloodmeal from an infected mammal. Once inside the fly, the bloodstream form trypanosomes transform, within a few hours into procyclic forms (Turner et al., 1988). In the case of *T. brucei* and *T. congolense* which have a midgut stage, the bloodstream form trypanosomes enter the endoperitrophic space, transform into procyclics before colonising the ectoperitrophic space where they establish midgut infections (Maudlin, 1991). From the midgut shelter, the trypanosomes migrate anteriorly towards the mouthparts with a temporary, but essential stopover at the proventriculus (PV) or cardia. While at the PV, the *T. brucei* species

undergoes asymmetric division yielding two forms of epimastigotes (one long and one short epimastigote) (Peacock et al., 2012a).

For *T. brucei*, it is the short epimastigote that invades and colonises the salivary gland while for *T. congolense* the proventricular form migrate to the proboscis and it is here that they differentiate into epimastigotes after attachment in the labrum of the proboscis (Sharma et al., 2008, Van Den Abbeele et al., 1999, Sakurai et al., 2009). The attached epimastigotes proliferate and differentiate into animal-infective metacyclic forms, which re-expresses a VSG coat and are therefore ready for life in the mammalian host (Tetley and Vickerman, 1985, Gray et al., 1981). This differentiation step known as metacyclogenesis, occurs in the salivary glands and labrum/hypopharynx in *T. brucei* and *T. congolense* respectively (Lloyd and Johnson, 1924, Thevenaz and Hecker, 1980). The reacquisition of a VSG coat is a typical feature of metacyclogenesis (Vickerman, 1985, Kolev et al., 2012, Tetley et al., 1987). In *T. vivax* there is no midgut stage (Figure 1.7) and it is thought that the procyclic stage occurs in the cibarium after which there is rapid transformation to epimastigotes which migrate to the proboscis where metacyclogenesis occcurs just as in *T. congolense* (Vickerman et al., 1988).

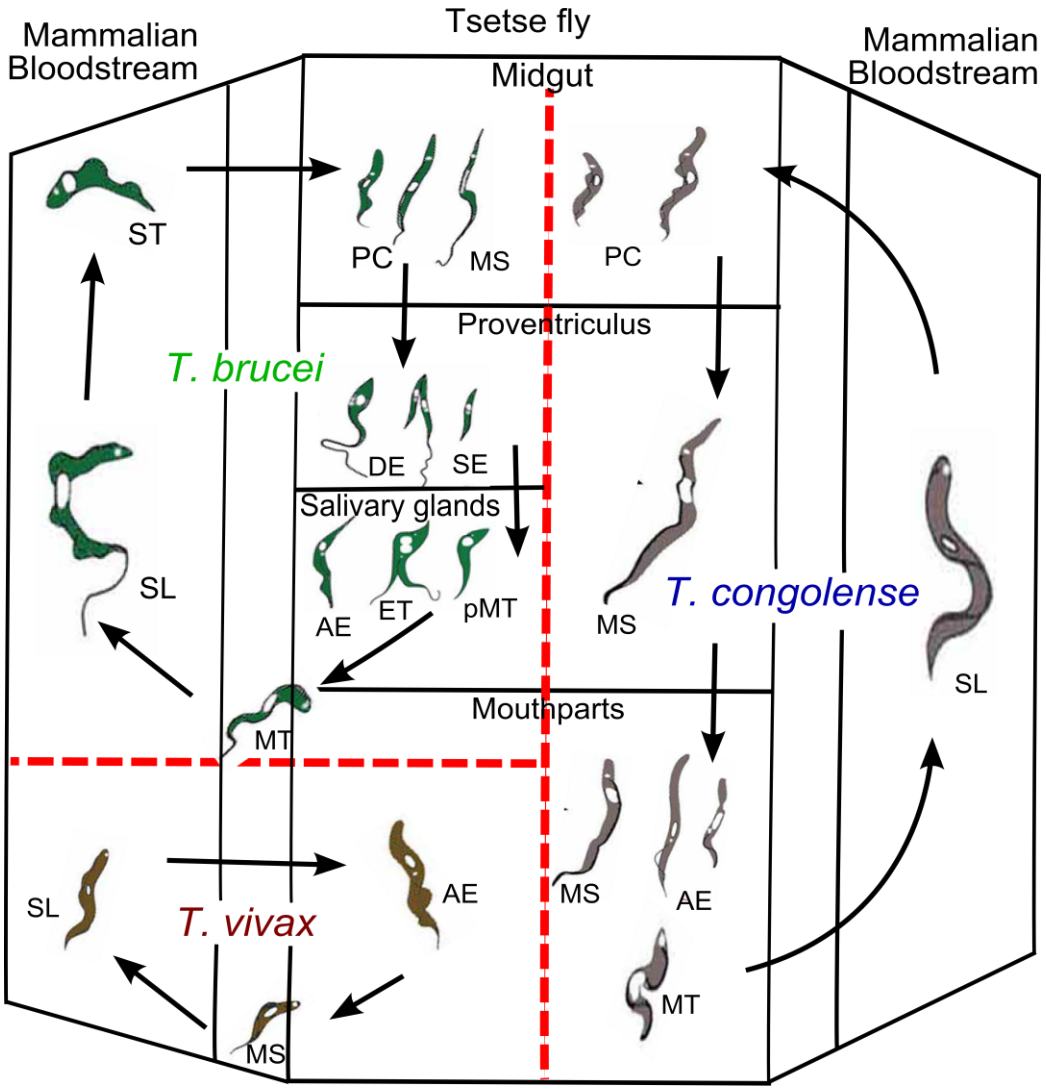

Figure 1.6 Life cycle of the three main types of African trypanosomes (*T. brucei, T. congolense* and *T. vivax*). Successive parasite stages found in the mammalian host and the tsetse fly vector are shown. SL: slender trypomastigote, ST: stumpy trypomastigote, PC: procyclic trypomastigote, MS: mesocyclic trypomastigote, DE: long dividing epimastigote, SE: short epimastigote, AE: attached epimastigote, ET: epi-trypo dividing epimastigote, pMT: pre-metacyclic trypomastigote, MT: metacyclic trypomastigotes (adapted from ILRI and Rotureau and Van Den Abbeele, 2013).

The infective metacyclic forms are injected into a mammalian host when an infected fly takes a bloodmeal. Inside the mammalian host the non-dividing metacyclics which have undergone cell cycle arrest while in the fly multiply by binary fission forming a heterogenous population of dividing slender forms and

non-dividing stumpy forms (Vickerman, 1985). The slender forms are seen during the ascending phase of parasitaemia while the stumpy forms which are irreversibly pre-adapted for life in the midgut of the fly are seen mainly when parasitaemia is at its peak (Matthews et al., 2004). The stumpy forms are also equiped with machinery to deal with the environmental shock (having been removed from a seemingly protective and well buffered environment to a harsh and harmful one) that these parasites will encounter in the digestive milieu of the insect midgut if taken up in a bloodmeal by the tsetse fly (Nolan et al., 2000). It is these stumpy forms that re-establish the life cycle by initiating differentiation into the replicative procyclic forms in the gut of the fly with the VSG surface coat being replaced by procyclins (Fenn and Matthews, 2007).

1.6 Genetic exchange in African trypanosomes

Genetic exchange is not an obligatory process in the life cycle of trypanosomes, but it can however occur between the midgut and the salivary glands stages of the life cycle (Peacock et al., 2011). The mechanism of genetic exchange is not fully understood but it is thought to be a truly Mendelian process that involves meiosis (Gibson and Stevens, 1999). Although it is not easy to identify the sexual stages, genetic exchange is known to occur in the laboratory between croses of *T. brucei* in the tsetse fly (MacLeod et al., 2005) and this has been demonstrated by co-infecting flies with red and green fluorescent parental trypanosomes leading to the production of yellow fluorescent hybrids in the salivary glands (Gibson et al., 2008). Also measurement of nuclear DNA contents revealed haploid promastigote-like cells relative to diploid metacyclics (Peacock et al., 2014).

In the field, if tsetse flies ingest more than one strain of trypanosome, there is the possibility of genetic exchange occuring between the two strains leading to an increase in genetic diversity among the African trypanosomes. Although the rate at which genetic exchange occurs in the field is low its implications for the epidemiology and control of trypanosomiasis could be far reaching (Gibson and Stevens, 1999). It could lead to the spread of important traits such as drug resistance and virulence since these can be transferred between pathogen

strains (Peacock et al., 2011). The use of chemotherapy in combination with other control measures such as vector control and systematic screening of pataients in at-risk areas have contributed to the recent success in reducing the number of cases (Nimmo, 2010). With no vaccine currently available and with few treatment options, the emergence of drug resistance is a major threat to the control and eradication of the disease (Baker et al., 2013).

1.7 Immune evasion

Unlike most pathogens that cause chronic infection, African trypanosomes do not seek refuge inside the cell, but rather they live extracellularly in their mammalian host and are therefore constantly bathed by the host immune defences. The human serum contains a trypanolytic factor that destroys *T. b. brucei* but not the human-infective subspecies *T. b. gambiense* and *T. b. rhodesiense* (Pays et al., 2006). Being highly immunogenic, they are prone to antibody-mediated immune response and therefore elicit immunological response from their mammalian host as well as complement in the blood of their mammalian host (Schwede and Carrington, 2010). Ironically, these parasites are able to maintain a chronic infection in their mammalian host despite these challenges.

African trypanosomes have developed several mechanisms to avoid being destroyed by the host immune system. First, the parasite population can withstand the host immune response as result of their ability to constantly change the major constituent of their cell surface, the variant surface glycoprotein (VSG) – a phenomenon called antigenic variation (Hall et al., 2013). Second, the compact surface coat which is mainly VSG, protects the invariant surface proteins from the effects of the immune system and prevents complement activation (Ferrante and Allison, 1983). Third, there is a rapid and effective internalisation of the immune complex formed as a result of the binding of antibody to VSG leading to the removal of host antibodies from the cell surface while the VSG is recycled and brought back to the surface (Engstler et al., 2007).

Finally, through mechanisms which are not fully understood, trypanosomes are able to modulate host immune responses to their advantage enabling them to maintain infection while at the same time allowing their host to survive for a period of time thereby enhancing their continued transmission (Mansfield and Paulnock, 2005). The parasites are capable of destroying the host's immunological memory through the abrogation of B-cell homeostasis while in the mammalian host, enabling them to evade immune reactions of the mammalian host thereby enabling the parasites to maintain a chronic infection (La Greca and Magez, 2011). During the course of trypanosome infection, there is a build-up of antibodies against the VSG that is already expressed which eventually leads to the clearance of the VSG and inevitable destruction of parasites expressing that particular VSG. However, prior to this, some parasites have already switched their VSG surface coat and are able to survive the onslaught of the antibodies and proliferate to form a new population of parasites that are subsequently recognised by the host antibodies and destroyed, and the cycle continues (Schwede and Carrington, 2010). By this mechanism African trypanosomes are able to pre-empt host specific immune responses, which enables them to maintain infection despite attempts by the host to eradicate them (Hall and Plenderleith, 2014).

1.7.1 Antigenic variation

The surface of the bloodstream form of African trypanosomes are covered with a monolayer of single VSG species (Pays, 2006). They are activated in the metacyclic stage of the parasite in the tsetse fly salivary glands (Tetley et al., 1987), though heterogeneous at this stage and remain active throughout the bloodstream stage until they are inactivated in the midgut of the tsetse fly after being ingested by the later (Horn, 2014). The fact that the VSG genes are activated just before the parasite leaves the salivary gland of the fly underlines the importance of the VSG for the continuation of its life cycle in the mammalian host. The genetic or epigenetic changes that result in the expression of a different VSG and the ability to ensure the activation of only a single VSG

through monoallelic expression of the active VSG gene are important for antigenic variation to occur (Schwede and Carrington, 2010).

The switching process in antigenic variation is stochastic rather than deterministic since it does not depend on host immune responses, however, the observed hierarchy of VSG expression is as a result of antibody selection (Morrison et al., 2009) (Figure 1.8). It also occurs spontaneously, having been observed in culture (Horn and Cross, 1997, Doyle et al., 1980) and in immunodeficient animals (Magez et al., 2008). VSG switching therefore occurs in anticipation of immune reaction and is controlled by a series of discrete events leading to the formation of a resident population of parasites displaying an abundant array of antigen (Hall and Plenderleith, 2014).

It has been argued that the first peak of parasitaemia is controlled by a combination of growth arrest as the density of the population increases and host immunity (Morrison et al., 2005, Lythgoe et al., 2007). Subsequently, as the infection progresses, there is a sequential expression of VSGs (Figure 1.8) as a result of mounting antibody responses which picks out the VSGs that are currently activated (Morrison et al., 2005). Switching of VSGs can occur in two ways: (i) transcriptional switching which does not require genetic rearrangement, rather as one of the multiple expression sites (ESs) is transcriptionally silenced another is activated. How this occurs is not fully understood but the two processes are not connected and are thought to involve several processes that lead to the rapid silencing of an old ES and complete activation of a new one (Figueiredo et al., 2008a), (ii) recombinatorial switching which typically occurs by gene conversion in which a silent VSG gene is copied into an active expression site (Horn and McCulloch, 2010). In addition to the VSGs that are present in the ESs, there are thousands of silent (inactivated) VSG genes and pseudogenes present in the subtelomeres forming a massive archive of VSG genes (Marcello and Barry, 2007). In natural infections recombination rather than transcriptional switching predominates in antigenic variation giving the parasite the ability to draw from its huge VSG archive by copying and pasting a different gene into an active ES (Robinson et al., 1999, Horn and McCulloch, 2010).

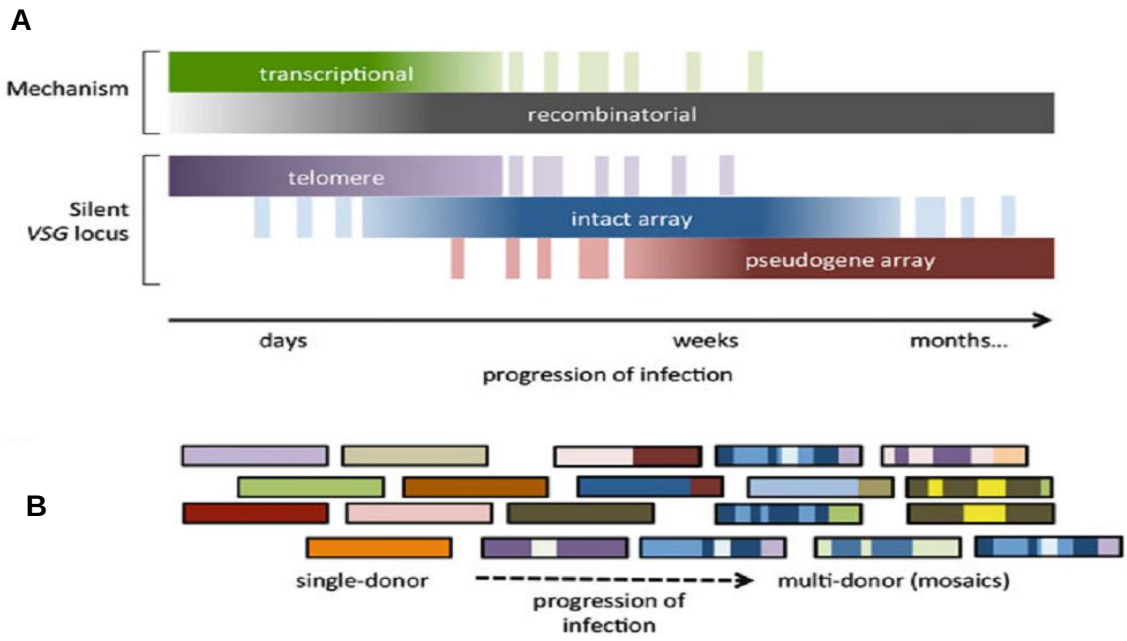

Figure 1.7 VSG switching hierarchy in *T. brucei*. (A) For each process, intensity of shading indicates the relative importance in activating novel VSG over the course of infection. Recombinatorial switching assumes prominence over transcriptional switching beyond the early stages of infection. Telomere-resident VSG in silent ES and minichromosomes are activated more readily than those in the VSG arrays. Pseudogenic VSG require low-probability segmental gene conversion events to be accessed and tend not to appear until the chronic stage of infection in the form of mosaic VSG. (B) Expressed VSGs are diverse, and mosaic VSGs become increasingly predominant as infections progress and immunity neutralises readily activated single-donor VSG. Shaded rectangles are a stylised representation of expressed VSG that might be sampled at different points of infection. Different shades represent different donors (Morrison et al., 2009, Hall and Plenderleith, 2014).

A feature of antigenic variation at the population level which helps the parasites to prolong infection in the host, is that the expression of VSGs is done in a hierarchical manner (Figure 1.8), with some VSGs being expressed at the early stage of infection, others are expressed midway during infection and yet others are expressed only during late infection (Barry, 1986, Barry and Turner, 1991, Gray, 1965). Some VSGs are more readily activated than others and are therefore expressed early on during an infection but can still be seen later in the course of an infection since they are still likely to be activated; but the problem is that parasites expressing these VSGs are prone to be destroyed if the antibodies

initially raised against them are still active (Morrison et al., 2005). Once the host immune responses destroy the easily-activated VSGs, those that are not easily activated because they require complex mechanisms to become active are then brought into play (Hall and Plenderleith, 2014). VSGs which appear late during infection are the incomplete or pseudogenes which need to be assembled by segmental gene conversion into mosaics and therefore appear late during infection (Roth et al., 1989). However, the pattern of the hierarchy is not rigid but rather flexible since the early VSGs can be interchanged with late VSGs (Laurent et al., 1984).

1.7.2 The trypanosome cell surface

The outer covering of the cell membrane of bloodstream trypanosomes are unusual in that it is covered predominantly with a glycosylphosphatidylinositol (GPI)-anchored protein, the VSG rather than the single hydrophobic transmembrane helix (Ferguson et al., 1988, Mehlert et al., 2002). Over 95% of the trypanosome external cell surface proteins and about 15% of the total cell protein is made up of VSG which forms approximately five million dimers per cell (Schwede and Carrington, 2010). The choice of a GPI-anchor comes with some advantages such as: (i) it enables the parasite to achieve a higher packing density on the cell surface without congesting the cytoplasmic face of the cell membrane and this is important since the VSG is packed on the surface at the highest possible density (Schwede and Carrington, 2010), (ii) it helps in VSG trafficking and recycling because without a GPI-anchor, VSG is rapidly delivered to the lysosome where it is degraded (Triggs and Bangs, 2003), (iii) GPI-anchor is essential for surface expression of VSG since in its absence the VSG is not expressed on the cell surface but is mislocalised to non-lysosomal compartments near the flagellar pocket (Bohme and Cross, 2002), (iv) Differentiation from bloodstream to procyclic form requires the rapid removal of the VSG coat by the synergistic action of the GPI-specific phospholipase C and a zinc metalloprotease (Grandgenett et al., 2007).

VSGs are also N-glycosylated, though the number of N-glycosylation sites vary between VSGs (Mehlert et al., 2002). It has been suggested that glycosylation

may enhance the volume of VSGs and thus its function as a protective coat (Blum et al., 1993). The VSG dimer forms a thick coat of about 12 to 15 nm that is attached perpendicularly to the cell surface (Vickerman, 1969) and physically blocks the binding of immunoglobulins to the cell surface (Schwede and Carrington, 2010). It has also been shown that antibodies cannot recognise VSG epitopes beyond 8 nm (Hsia et al., 1996).

1.7.3 Endocytosis, membrane recycling and sorting of antibody-bound VSGs

The lack of transmembrane domain allows the VSG coat to move freely across the surface of the parasite and there is evidence that the fluidity of the VSG coat helps in the removal of immunoglobulins that are bound to the VSGs especially at low to moderate antibody concentration thereby augmenting the immune evasion mechanisms of the parasite (Engstler et al., 2007). The immunoglobulin-VSG immune complex moves more rapidly than free VSG (Schwede and Carrington, 2010). The directional movement of the Ig-VSG complex towards the flagella pocket at the posterior end of the parasite is aided by the hydrodynamic flow acting on the trypanosome as it swims and this drags the Ig-VSG complex as the cell swims forward (Schwede and Carrington, 2010).

1.8 The vector (Tsetse fly)

Tsetse flies belong to the order *Diptera* (two-winged fly), family Glossinidae and genus *Glossina*. There are 33 extant taxa with 22 species and subspecies and all are restricted to sub-Saharan Africa (Elsen et al., 1990, Leak, 1999). Three groups or subgenera are recognised within the *Glossina* genus: *Morsitans (Glossina* Wiedemann), *Palpalis* (Nemorhina Robineau-Desvoidy) and *Fusca* (Austenina Townsend). They occur in a broad range of habitats distributed discontinuously throughout their range in the sub-Saharan continent with each group occupying a relatively specific habitat (Leak, 1999). The *Morsitans* group are found mainly in Savannah, woodlands and low land forests in East Africa and near rain forests in West Africa.

Flies of the *Palpalis* group are found in West Africa, mainly in the lowland rainforests, particularly along rivers and streams (Rogers, 2004). *Fusca* group of flies are found in West Africa where they live in moist forests and savannah (Caljon et al., 2014), some species such as *G. brevipalpis* are found discontinuously in East Africa, Democratic Republic of the Congo, and Mozambique (Benoit et al., 2015). In terms of host preference, flies in the *Palpalis* group are strongly anthropophilic while flies in the *Morsitans* and *Fusca* groups are more zoophilic (Benoit et al., 2015). Unlike mosquitoes, both male and female flies are obligate blood feeders and feed on vertebrate blood every 3-4 days (Leak, 1999).

1.8.1 Classification

Tsetse flies are classified into a single genus, *Glossina* and classification is based largely on morphological differences in the structure of the genitalia, geographical distribution and some bio-ecological features, but also on some external features such as colour, shape of antennae and presence of bristles on thoracic pleura (Newstead, 1911), of the male genital armature. Three groups or subgenera are identified within the genus - *Nemorhina* (*Palpalis* group)*, Glossina* (*Morsitans* group)*,* and *Austenina* (*Fusca* group) (WHO, 2013a). The species in the *Palpalis* group which are the main vectors of HAT in West and Central Africa are found in vegetations close to water, in mango and banana platations, as well as in peri urban areas (Robays et al., 2004). The *Morsitans* group are found mainly in savannah woodland and dense or clear forests mainly linked to the presence of wild fauna and cattle and species in this group are the main vectors of African animal trypanosomiasis, although some are involved in the transmission of *T. b. rhodesiense* (WHO, 2013a). Species in the *Fusca* group are bigger in size than those in both the *Palpalis* and *Morsitans* groups and they live in forest belts in East and Southern Africa.

1.8.2 Reproductive physiology and anatomy

Tsetse flies exhibit an unusual mode of reproduction among insect disease vectors. They are viviparous because they bring forth live young which has developed in the uterus of the female. Tsetse flies reproduce by adenotrophic viviparity (a form of matrotrophic viviparity) in which the female does not lay eggs but nurtures a single larva at a time within its uterus with nutrients secreted through glands until a mature larva is larviposited (Benoit et al., 2015). Tsetses also have a low reproductive rate, producing no more than eight to ten offsprings during its life span (Attardo et al., 2006). Unlike many higher Dipterans, their reproductive tracts have undergone considerable modifications (Tobe and Langley, 1978), along with other members of Hippoboscoidea, to allow for complete intrauterine larvae development, which include reduction of the number of ovarioles per ovary to two compared to ovaries from other dipterans, the uterus which is greatly expanded, is muscular and supplied with numerous trachea, and the possession of accessory or milk glands that connects to the uterus (Attardo et al., 2006, Pellegrini et al., 2011, Tobe and Langley, 1978).

1.8.3 Life Cycle of Tsetse Flies

Female tsetses produce a single larva per gonotrophic cycle which begins when females emerge from the puparium (Tobe and Langley, 1978). The first oocyte begins development (at the right ovary) following the emergence of teneral females from the puparium. During mating, which occurs 3-5 days post eclosion, sperm are deposited in the spermatheca where they are stored until fertilization during ovulation and the first ovulation occurs at about 10 days after emergence. This is followed by intrauterine embryogenesis and larvigenesis. From embryogenesis to larvigenesis takes approximately 10 days with the former lasting 3-4 days while the later takes 5-6 days respectively (Saunders and Dodd, 1972, Tobe and Langley, 1978). The second oogenesis commences within the left ovary during intrauterine embryonic and first instar larval development in the right ovary and is completed before the completion of the first larval development. Female tsetses deliver their first offspring approximately 20 days after emergence following the deposition of larvae which develops into pupae

within 1-2 hours and the adult emerges after 30 days (Tobe and Langley, 1978). Ovulation occurs at the left ovary 20-35 minutes after the deposition of the first larva and the second larva is deposited 9-10 days after the first. The availability of blood meal and type of host, exposure to conditions that interfere with oogenesis or milk production, as well as environmental factors affects the duration of the developmental cycle (Tobe and Langley, 1978, Saunders and Dodd, 1972). Oogenesis is shared between the two ovaries during each gonotrophic cycle (Saunders, 1960, Saunders, 1961).

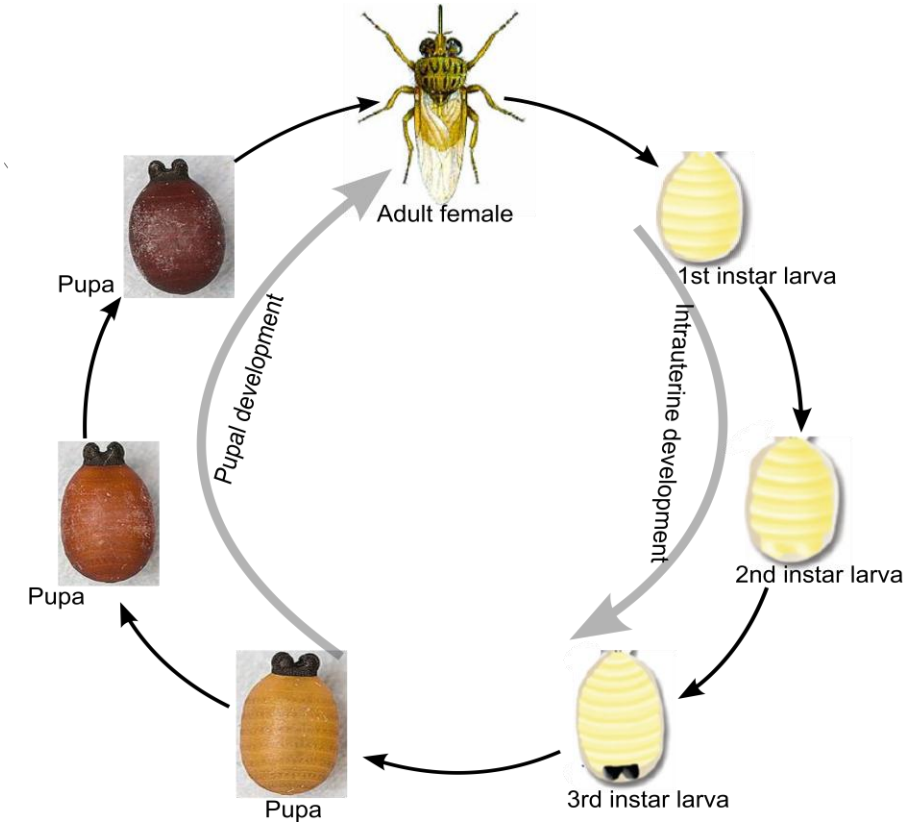

Figure 1.8 Life cycle of *Glossina morsitans morsitans* female under optimal and nutritional conditions. The different stages of intrauterine and pupal developments are shown (adapted from Benoit et al., 2015).

Pregnant females carrying a mature oocyte do not ovulate until after parturition or abortion. This shows that ovulation in tsetse is regulated and coordinated by the mating and pregnancy status of the fly (Benoit et al., 2015). Two major events regulate ovulation, namely mating status and oocyte development and

mating status is determined by the stimulus generated from a lengthy copulation which lasts between 90-120 minutes (Chaudhury and Dhadialla, 1976, Saunders and Dodd, 1972). However, the mating status of a female does not trigger ovulation because for most females mating does not occur until after 2-3 days following emergence from the pupae and will not ovulate until their first oocyte is mature at 8-10 days posteclosion (Tobe and Langley, 1978). The presence of a mature oocyte in mated females which is thought to release an ovulation-stimulation factor into the haemolymph seems to be the trigger for ovulation (Robert et al., 1984, Foster, 1974, Chaudhury et al., 1981). After ovulation the embryo develops in the uterus and goes through three larval instars before finally being deposited as a fully developed third instar larva which burrows into the ground and pupariates (Buxton, 1955, Denlinger and Ma, 1974, Moloo, 1971b) (Figure 1.8).

During larvigenesis the fly nourishes the larva by supplying milk from an accessory gland that has undergone extensive physiological modification to cater for a larva that increases over a hundred-fold in dry mass in the course of six days (Langley and Pimley, 1975, Denlinger and Ma, 1974). Milk secretions not only provide nourishment for the developing larva, it is also the means through which microbial symbionts are transferred to their offspring during intrauterine development (Ma and Denlinger, 1974, Balmand et al., 2013). It also ensures that the microbiota is passed on with the highest fidelity preventing the exposure of the larvae to unwanted microbes (Weiss et al., 2011). Starved flies were found to be more permissive to trypanosome infection.

1.8.4 Tsetse immune system

Insects represent one of the most successful and largest groups of animals, occupying virtually all ecological niches and as a consequence contend with a large number of pathogens (Vilmos and Kurucz, 1998). It is no surprise therefore that they have evolved a complex and effective innate immune system to deal with the threat of these pathogens. The insect immune mechanism is a complex and tightly regulated system which has the ability to recognise, control or eliminate the invading organisms in a way that strikes a balance between

mounting anti-microbial effector molecules and controlling pathogenicity (Caljon et al., 2014). The tsetse fly, like all insects is a heaven for microorganisms ranging from commensals and symbionts to opportunistic microbes and pathogens (Caljon et al., 2014). The first line of defence is the cuticle and peritrophic matrix which act as physical barriers against invading microorganisms (Lehane, 1997).

The insect immune response is divided into cellular and humoral mechanisms. Cellular immune response includes phagocytosis and encapsulation, while humoral or molecule-based mechanisms involve synthesis and secretion of antimicrobial peptides (AMPs), lectins, reactive oxygen species (ROS), proteases and nitric oxide, coagulation and melanisation cascades (Boulanger et al., 2002). Humoral immune responses have been shown to be involved in the clearance of trypanosomes, but there is no documented evidence of a cellular involvement in trypanosome clearance since trypanosomes do not enter the haemocoel (Caljon et al., 2014). An important aspect of tsetse immunity is the expression of several AMPs (Boulanger et al., 2002). Also the nutritional state of the fly at the time of blood meal has an effect on the ability of the fly to withstand trypanosome infection, and starvation correlated to low expression of the antimicrobial peptides attacin and cecropin and increased susceptibility to trypanosome infection (Akoda et al., 2009). Also midgut lectins are thought to play a role in the determination of parasite establishment and infection of tsetse because feeding lectin inhibitory sugars to tsetse significantly increases trypanosome midgut infection (Maudlin and Welburn, 1987, Ibrahim et al., 1984, Mihok et al., 1992, Welburn et al., 1994).

Reactive intermediates of oxygen and nitrogen such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are also involved in innate immune response in tsetse. Adding antioxidants to the infective blood meal increase susceptibility to trypanosome infection confirming the trypanocidal activity of ROS (MacLeod et al., 2007b). Also ROS-induced stress leads to the production of trypanocidal nitric oxide (NO) by nitric oxide synthase (NOS) in the gut (Bogdan, 2001), which in turn triggers AMP production in the fat body, thus representing an important link in organ-organ immunological communication (Wu et al., 2012). It has also been suggested that hydrogen peroxide (H_2O_2) may be

involved in eliminating trypanosomes early in the infection process because H_2O_2 level was found to be significantly increased in flies that have been fed infective blood meal (Hao et al., 2003).

1.8.5 Signaling pathways controlling trypanosome infection in tsetse

In insects, three immunity signaling pathways (Toll, IMD and Jak/Stat) are known to be involved in immune responses against invading pathogens. Following the induction of these signaling pathways is the activation of transcription factors and the subsequent expression of antimicrobial peptides (Kingsolver and Hardy, 2012). The inducible expression of antimicrobial peptides is overseen by the Toll and immune deficiency (IMD) pathways (Tanji et al., 2007), and it has been shown that without these two pathways, flies become more susceptible to microbial infections, even to those that are ordinarily non-pathogenic because of the failure to induce antimicrobial peptide genes (Gottar et al., 2002, Tzou et al., 2002, Vodovar et al., 2005). The Toll pathway is activated by fungal and gram-positive bacterial infections while the IMD pathway is activated by gram-negative bacterial infection (Lemaitre, 2004). Microbe detection and subsequent response of the insect innate immune system involves a multistep process which occurs as a result of the direct contact between the insect pattern-recognition receptors such as the peptidoglycan recognition proteins (PGRPs), for bacterium recognition and pathogen-associated molecular patterns (PAMPs) (Beschin et al., 2014).

In tsetse, the IMD pathway and PGRP-LB is known to control trypanosome infection (Beschin et al., 2014). Stimulation of tsetse IMD pathway through bacterial challenge before trypanosome infection increases the fly's resistance to trypanosome infection (Hao et al., 2001). Three antimicrobial peptide genes, *defensin, attacin, and cecropin* are differentially expressed when tsetse flies are stimulated by bacteria or different life stages of trypanosomes, suggesting that the immune machinery of tsetse are able to discriminate between specific molecular signals from different pathogens (Hao et al., 2001). Also newly emerged flies presented a weakened immune response when challenged with trypanosomes, in contrast to adult refractory flies in which there were higher

levels of genes associated with the IMD pathway (*attacin* and PGRP-LB) (Weiss et al., 2013).

The establishment of midgut infection was found to be significantly higher in tsetse when *attacin* or the IMD transcriptional activator *relish* was suppressed through RNAi knockdown, implying that IMD-regulated AMPs play a role in resisting trypanosomes in the midgut of tsetse flies (Hu and Aksoy, 2006). Neither the parasite-specific components nor the tsetse fly recognition proteins involved in triggering the IMD pathway is not fully known, but it must be said that several factors are involved in the recognition and subsequent response of a typical insect like tsetse, to microbial infection and requires direct contact between the parasite-specific components in the form of pathogen-associated molecular patterns (PAMPs) and tsetse pattern-recognition receptors like PGRPs (Beschin et al., 2014).

The *Glossina* genome contains six PGRP genes made up of four long and two short subfamilies (Attardo et al., 2014). Tsetse PGRP-LB is involved in regulating the density of bacterial symbiome as well as controlling trypanosome infection. This is evident when, following the depletion of PGRP-LB, the IMD signalling pathway is activated which leads to the synthesis of AMPs that reduces the density of *Wigglesworthia* with a surprise increase in trypanosome infection rate despite the synthesis of AMPs (Wang et al., 2009). When both PGRP-LB (Toll) and IMD pathway are suppressed, flies become more susceptible to trypanosome infection suggesting that both may be acting synergistically. Moreover, trypanosome infected adult flies have significantly lower PGRP-LB expression levels than self cured flies (Wang et al., 2009). The PGRP-LB protein has been shown to be trypanocidal (Wang and Aksoy, 2012). It is also maternally transferred to the larva through the mother's milk, during intrauterine development and the amount of PGRP-LB transferred is directly proportional to the density of *Wigglesworthia* in the milk gland and it has been suggested that the level of trypanosome resistance exhibited by teneral (young and unfed) flies is directly linked to the amount of PGRP-LB present in the midgut (Weiss et al., 2013, Wang and Aksoy, 2012).

1.9 Tsetse flies as cyclical vectors of trypanosomes

Tsetse flies (both males and females) are the only cyclical vectors of African trypanosomes and therefore occupy an important position in the epidemiology of African trypanosomiasis. The cyclical transmission of African trypanosomes by tsetse flies is a complex phenomenon that involves the parasites undergoing a developmental cycle in the fly resulting in a final infective stage that is transmitted to a new mammalian host during a blood meal by the fly. An interplay between various factors determines the susceptibility or otherwise of the flies to trypanosome infection. The cyclical transmission of trypanosomes by tsetse flies is a complex process that is dependent on a number of factors such as: (1) availability of an infected tsetse fly (vector), (2) the ability of the parasite to successfully undergo complete developmental cycle leading to the production of mammalian infective metacyclics in the fly, (Sbicego et al., 1999, Van Den Abbeele et al., 1999) and (3) the injection of the parasite to a new mammalian host during a blood meal by the infected fly.

Consequently, three critical stages in the cyclical transmission of African trypanosomiasis can therefore be identified as follows: (i) invasion of the midgut, (ii) establishment of infection/multiplication of parasites in the midgut, and (iii) maturation of parasites in the mouthparts (Vickerman et al., 1988, Maudlin and Welburn, 1994, Van Den Abbeele et al., 1999). Vector competence which is the ability of the fly to acquire trypanosome infection, favour the maturation of the parasite, and transmit it to a mammalian host is therefore vital to the transmission of African trypanosomiasis.

A better understanding of the molecular nature of tsetse-trypanosome interactions offers potential opportunities for the development of fly-based control strategy. Different models have been designed to explain the mechanisms underlying trypanosome transmission by tsetse flies (Gouteux and Artzrouni, 1996, Rogers, 1988). However, the phenomenon of tsetse refractoriness to trypanosome infection remains poorly understood.

1.10 Tsetse-trypanosome molecular interactions

It is an established fact that tsetse flies are naturally resistant (refractory) to trypanosome infection; even under ideal laboratory conditions the proportion of flies that harbour mammalian infective forms of the parasite is low (Aksoy et al., 2003, Rio et al., 2004). Once an uninfected tsetse fly acquires bloodstream form (BSF) parasites through a blood meal from an infected mammalian host, an intriguing and intricate molecular war ensues between the trypanosomes and their tsetse fly vector, each having devised mechanisms to mislead and outwit each other in order to come out triumphant at the end of the molecular dialogue. Once in the gut of fly the parasites are faced with a host of physical and immunological defence mechanisms (Weiss et al., 2013), and a myriad of immune molecules that are produced by the fly to prevent trypanosomes from establishing infection in the midgut. The molecular cross-talk between trypanosomes and their tsetse fly vector are of considerable importance in the epidemiology of African trypanosomiasis. The major conditions that influence fly infection rate are discussed below.

1.10.1 Factors that influence the outcome of tsetse-trypanosome interaction

There are various factors associated with the fly that interferes with trypanosome colonisation of the midgut and subsequent maturation in the salivary glands or mouthparts. The probability that a tsetse fly will become infected after taking a blood meal from an infected mammal will depend on the vectorial capacity (ability to become infected and support the complete developmental cycle of the parasite) of the vector (De Deken, 2013). The main factors that influence the outcome of the interaction between tsetse and trypanosomes are discussed below.

1.10.1.1 Fly age at the time of infective blood meal

Tsetse flies are generally believed to be more susceptible to trypanosome infection if their first blood meal is infected. In order words, newly emerged flies

that have not yet had a blood meal after eclosion are most susceptible to trypanosome infection – the so-called teneral phenomenon (Distelmans et al., 1982, Welburn and Maudlin, 1992, Mwangelwa et al., 1987, Wijers, 1958). The susceptibility of newly emerged flies to trypanosome infection was found to be evident in both sexes, although males were found to have a higher infection rate than females in flies that had infective blood meal within 32 hours after eclosion (Makumyaviri et al., 1984, Wijers, 1958, Distelmans et al., 1982). Because newly emerged flies are more susceptible to trypanosome infection, there is the assumption that, in its teneral state, the fly is not yet well "equipped" to resist the invading parasites. A number of theories have been put forward to explain the teneral phenomenon. For example, the role of the peritrophic membrane (Lehane and Msangi, 1991), the role of midgut lectins (Maudlin and Welburn, 1987, Welburn et al., 1989, Yoshino and Vasta, 1996), the role of antitrypanosomal factors such as midgut trypanolysin and trypano-agglutinins released during trypanosome infection (Molyneux and Stiles, 1991).

1.10.1.2 Role of the peritrophic matrix

The peritrophic matrix in the midgut of insects is a protective, chitinous layer that protects the midgut epithelium from invading microorganisms and parasites and the abrasive action of digestive enzymes. In tsetse, the PM is produced by a group of specialised cells present on the proventriculus (PV) in the anterior section of the midgut and secreted continuously as an unbroken, concentric, "sleeve-like" structure (Type II) (Tellam et al., 1999). It has been assumed that in order to establish infection in the midgut, trypanosomes must cross the PM to enter the ectoperitrophic space where they multiply and continue the process of differentiation (Freeman, 1973, Ellis and Evans, 1977, Gibson and Bailey, 2003). In as much as the PM forms a physical barrier to keep out pathogens from blood meal (Lehane, 1997, Hegedus et al., 2009), it has been suggested that the PM can also play an indirect role in trypanosome establishment since, in the absence of a robust PM, the gut epithelia is prematurely exposed to antigens from invading trypanosomes and therefore elicits an early immune response

which does not surprisingly confer long term protection from trypanosome establishment (Weiss et al., 2013).

It has been demonstrated that the length of the fly's PM determines the susceptibility or otherwise of tsetse to trypanosome infection because the shorter the PM, the more susceptible the flies are to trypanosome infection and vice versa, since midgut infection prevalence is lower in adult flies with fully formed PM compared to teneral flies whose PM are not fully formed (Walshe et al., 2011b). The molecular mechanisms underlying the involvement of the PM in tsetse refractoriness to trypanosome infection is yet to be unravelled, but it has been suggested that the PM regulates the timing of host immune induction following trypanosome challenge as well as acting as a physical barrier between ingested microbes and the immune-reactive gut epithelia (Weiss et al., 2013). A model depicting the role of tsetse PM in modulating trypanosome infection outcome is depicted in Figure 1.9. Although the PM forms a physical barrier between trypanosomes and the gut epithelia, some parasites succeed in breaching this barrier through a mechanism that is yet unknown, but are subsequently eliminated in a majority of flies within the first three days as a result of a robust immune reaction mounted by the fly (Gibson and Bailey, 2003). However, parasites that can survive the initial immune responses go on to proliferate in the tsetse's gut even in the presence of AMPs indicating that tsetse-trypanosome interaction is a much more complex process (Hao et al., 2001).

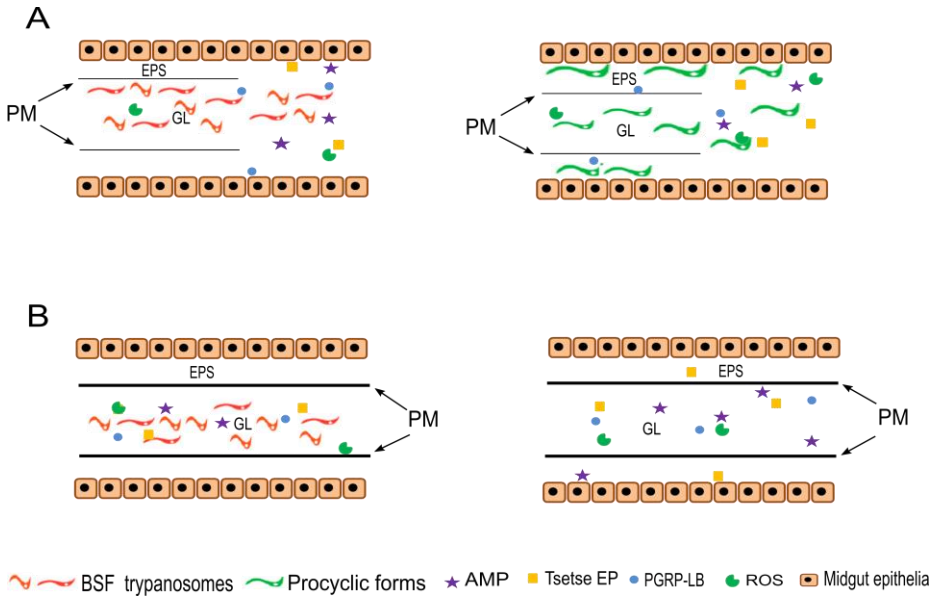

Figure 1.9. Models depicting the role of tsetse PM in modulating the immune response following challenge with trypanosomes.

The presence of an intact PM in the gut separates the luminal contents (including trypanosomes) from immune-competent epithelial cells. **(A)** In teneral flies with immature PM are more susceptible to trypanosome infection despite the early exposure of the parasites to epithelial immune response. **(B)** Adult flies with fully formed (mature) PM covering the entire gut succeed in clearing the invading parasites (Adapted from (Weiss et al., 2014).

1.10.1.3 Fly species and genetic variation between individual flies

Susceptibility to trypanosome infections varies between tsetse species. For example, tsetse flies belonging to the *morsitans* group are relatively more susceptible to trypanosome infection compared to *palpalis*-group flies (Harley and Wilson, 1968, Moloo and Kutuza, 1988b, Moloo and Kutuza, 1988a, Jordan, 1964, Roberts and Gray, 1972). Even within the same species susceptibility to trypanosome infection may vary. Salmon eye colour mutation in *G. m. morsitans* was found to be associated with increased susceptibility to *T. congolense* infection (Distelmans et al., 1985). Refractoriness in tsetse is not an all-or-nothing phenomenon as it occurs in the proportion of flies infected (Dyer et al., 2013). This is demonstrated in the pattern of selection of lines of *G. m. morsitans*

and *G. m. centralis* susceptible or refractory to *T. congolense* infection (Maudlin and Dukes, 1985, Moloo et al., 1998). Since susceptibility to midgut infection was solely maternally inherited, with no input from the male, it is said to be extrachromosomally inherited (Moloo et al., 1998, Maudlin, 1982). The extrachromosomal factor was thought to be rickettsia-like organisms (RLOs) present in the midgut of tsetse (Maudlin and Ellis, 1985), which produces chitinase which in turn releases glucosamine thereby inactivating lectins (Maudlin and Welburn, 1988b). The RLOs were later identified as *Sodalis glossinidius* (Weiss and Aksoy, 2011). It should therefore be expected that tsetse selected for either susceptibility or refractoriness to *T. congolense* would not exhibit the same phenotype when tested with *T. vivax* since the latter's developmental cycle is restricted to the proboscis (Maudlin et al., 1986). However, this was not the case as the refractory lines were found to be more refractory to *T. vivax* and other trypanosome strains and species indicating that the extrachromosomal susceptible factor exerts its effects outside the midgut (Moloo et al., 1998). Selective elimination of *Sodalis* without affecting the obligate symbiont, *Wigglesworthia glossinidia* significantly reduces the vectorial capacity of tsetse (Dale and Welburn, 2001).

1.10.1.4 Sex of the fly

Among dipterans that are vectors of disease, tsetse fly is unusual because both male and female flies are obligate blood feeders and therefore capable of transmitting trypanosomes. However, males are known to be more susceptible to *T. brucei* infection than females (Dale et al., 1995, Maudlin et al., 1990, Moloo et al., 1992). Males are also known to show higher rate of infection maturation than females even though females have similar or higher rate of immature (midgut) infection (Distelmans et al., 1982, Mwangelwa et al., 1987, Maudlin et al., 1990). It has been proposed that the female tsetse SG environment may be more hostile to trypanosome establishment compared to their male counterparts leading to lower rates of SG infection in the former, though the molecular mechanisms responsible for this is not yet known (Peacock et al., 2012b). Since female tsetse relatively live longer than the males (Phelps and Vale, 1978), and

since *T. brucei* undergo a long developmental cycle in the fly, the females are more likely to be exposed to SG infection.

1.10.1.5 Variation between trypanosome species and strains

Different species and strains of trypanosome vary in their ability to establish infection in tsetse. Not all strains of *T. b. gambiense* were able to establish salivary gland infections in *G. palpalis* even though they established midgut infections in the flies, and the strains that failed to establish salivary gland infection were said to be non transmissible (Duke, 1930). However, strains of *T. b. rhodesiense* were always able to produce salivary gland infections in tsetse (Duke, 1933). Therefore, tsetse transmissibility varies between trypanosome species as well as between strains. Strains of the human pathogenic *T. b. rhodesiense* are less likely to produce mature infection in *G. m. morsitans* compared to its closely related *T. b. brucei* strains (Macleod et al., 2007a). Also virulent strains of *T. congolense* produced higher midgut infections than strains of moderate or low virulence (Masumu et al., 2006). Since *T. congolense* is believed to be monomorphic, existing only in one form, therefore no variation in infectivity would normally be expected. However, parasites from the acute phase of infection in mice produce higher rates of midgut infection in tsetse than those from the chronic phase, regardless of the level of parasitaemia (Masumu et al., 2010). Also differences on the ease with which trypanosomes develop in the fly have been observed between flies fed on infected mouse blood between 4 to 10 days post-infection, indicating that the rate of maturation of trypanosome in tsetse depends on the phase in the parasite's development in the mammalian host (Akoda et al., 2008).

1.10.1.6 Gut pH, proteases and lectins

In tsetse blood meal digestion is carried out in the digestive tract which can be divided into: the anterior midgut for storage of blood meal, middle portion for secretion of digestive enzymes, and the hindgut for blood meal digestion and absorption of nutrients. The ingestion of a blood meal by tsetse triggers the

secretion, in the midgut, of proteases (Imbuga et al., 1992b), trypanolysins (Stiles et al., 1990, Osir et al., 1999), lectins (Maudlin, 1991, Stiles et al., 1990) and other unidentified factors and the accumulation of these factors makes the environment very hostile to the ingested parasites, majority of which are destroyed within 3 days of being ingested (Stiles et al., 1990, Abubakar et al., 1995). The few parasites that survived enter the ectoperitrophic space where they establish midgut infections (Maudlin, 1985, Maudlin and Welburn, 1987). *T. b. brucei* were found to be capable of inhibiting tsetse midgut trypsin extracts (Imbuga et al., 1992a). In addition, a tsetse midgut lectin-trypsin complex was capable of inducing the transformation of the stumpy bloodstream forms to procyclic forms (Abubakar et al., 2006, Dean et al., 2009).

1.10.1.7 Blood meal species

The source of mammalian blood meal seems to play a role in determining the refractoriness or permissiveness of tsetse to trypanosome infection. In the laboratory, tsetse flies fed on infected goat or pig blood recorded more midgut infections than flies fed infected blood from other mammalian sources (Aksoy et al., 2003). When flies are fed PCF trypanosomes suspended in PBS-washed red blood cells containing heat-inactivated serum, midgut infection rates were observed to be high (Evans, 1979), suggesting that serum complement may be controlling trypanosome infection in tsetse. Also PCF trypanosomes are known to be susceptible to vertebrate complement *in vitro* (Walshe et al., 2011a). Despite the fact that trypanosomes in the midgut are exposed to constant contact with serum complement since the fly takes a blood meal every 48 hours, the parasites are still able to complete their life cycle in the fly. This implies that the parasites in the midgut of tsetse could have evolved ways to either circumvent or inactivate the action of complement.

1.10.1.8 Nutritional status

The nutritional state of the fly at the time of infective blood meal also affects its ability to fight trypanosome infection because flies subjected to extreme

starvation (>3 days after eclosion) were found to offer less resistance to trypanosome infection (Kubi et al., 2006). It has been demonstrated that nutritional stress leads to a reduction in the levels of the AMPs attacin, defencin and cecropin expressed in the fat body, which may lead to increased susceptibility to trypanosome infection in starved flies (Akoda et al., 2009). Although it is an established fact that newly emerged flies are highly susceptible to trypanosome infection, the ability of adult flies to acquire mature trypanosome infection is also relatively high, especially for *T. congolense* (Kubi et al., 2006). Starving young and adult tsetse flies prior to infection increases their susceptibility to trypanosome infection, suggesting that the mechanisms involved in the resistance of tsetse flies to trypanosome infection may have been undermined by starvation (Kubi et al., 2006). The authors argued that starvation depletes the fat body energy reserves leading to a possible suppression of the ability of the fly to mount a robust immune response. Also nutritional stress leads to a decrease in the expression of AMPs thereby altering tsetse immune response to trypanosome infection (Akoda et al., 2009).

1.10.1.9 Tsetse innate immune response in trypanosome infection

Upon infection, insects mount a rapid innate immune response that is made up of various molecules such as antimicrobial peptides (AMPs), haemocytes, and phenoloxidase-based melanisation (Brennan and Anderson, 2004, Hultmark, 2003, Lemaitre, 2004, Tanji and Ip, 2005). The sequencing of the genome of the tsetse fly (*Glossina morsitans*) has revealed that its innate immune system is similar to that of *Drosophila* (Kounatidis and Ligoxygakis, 2012, Attardo et al., 2014). The establishment of an endogenous microbiome during intrauterine larval development of tsetse is important for the development of a fully functional innate immune system later in the mature adult (Weiss et al., 2011, Weiss et al., 2012).

Reactive oxygen species (ROS)-mediated immune response in the alimentary tract of tsetse flies also modulate trypanosome infection outcome in the fly (Beschin et al., 2014). ROS which is produced by dual oxidase (Duox) exhibit oxidative antimicrobial activity in dipterans (Ha et al., 2005a) as well as

antiparasite activity (Kumar et al., 2003). They can also act as messenger molecules which help to evoke systemic immune response in the fat body as well as nitric oxide (NO) production through the activation of inducible nitric oxide synthase (iNOS). After ingesting trypanosomes during a blood meal, there is an increase in the level of hydrogen peroxidise in the midgut/proventriculus of the fly apparently in a bid to clear the invading trypanosomes early on during infection (Hao et al., 2003). Self-cured flies are known to mount a systemic oxidative stress response (caused by an increase in ROS) in the fat body suggesting a role for oxidative stress in refractoriness of tsetse to trypanosome infection (Lehane et al., 2008) as well as an up-regulation of iNOS and Duox, genes associated with ROS-mediated immunity (Weiss et al., 2013). Inhibiting the action of ROS by supplementing infective blood meal with antioxidants such as L-glutathionate and ascorbic acid, leads to an increase in midgut infection rate, thus supporting the view that ROS play a role in trypanosome clearance in the fly (MacLeod et al., 2007b). Oxidative stress has also been implicated in immune defences in Drosophila and *Rhodnius prolixus* (Ha et al., 2005b, Whitten et al., 2001).

The TsetseEP protein is another immune responsive protein which is present in the midgut, haemolymph, as well as salivary glands. Tsetse EP protein is named because of its possession of an extensive tandem repeat units of glutamic acid (E) and proline (P) which make up more than 40% of the amino acid residue (Haines et al., 2010), with the repeat units showing remarkable sequence identity to that of *T. b. brucei* EP composition (Vassella et al., 2001). Although not much is known about tsetse EP protein, the fact that it is constitutively expressed both in adult fat body and midgut and is upregulated following immune challenge (Haines et al., 2005). Also suppression of TsetseEP by RNAi leads to an increase in midgut trypanosome infection rate (Haines et al., 2010). Trypanosomes once inside the alimentary tract of the fly are met with stiff opposition mounted by the fly innate defence system already existing, albeit at a basic level, i.e., at pre-immune challenge level but is induced systemically (in the fat body) and locally in the form of epithelial response in the alimentary tract (Figure 1.10) (Beschin et al., 2014). These defensive mechanisms are deemed to be very effective since they succeed in eliminating the parasites in most cases

which is reflected in the low number of flies that are susceptible to trypanosome infection in the field.

The trypanosomes on their part have also devised some strategies to bypass the defensive mechanisms of the fly, for example, the BSF once in the midgut, switches its VSG surface coat to a new stage-specific coat – the procyclin, a move designed to curtail the effectiveness of the tsetse innate immune response (Güther et al., 2006, Pearson, 2001). Parasites that succeed in establishing infections in the midgut may be either resistant to tsetse immune responses or the host immune responses are induced in a such a way as to reduce parasite densities to reduce fitness cost to infected flies. It seems however, that the former is the case since during the course of infection in the fly's midgut, trypanosomes switch their surface coat from one composed of GPEET to one composed predominantly of immune-invasive EP proteins (Ruepp et al., 1997, Urwyler et al., 2005). The expression of a less antigenic surface coat may be an adaptation on the part of the parasites that enables them to curtail immune reaction from the fly thereby enabling them to survive in the immunocompetent gut of the fly. This adaptation may have arisen because of the long-term host-parasite co-evolutionary process between tsetse and trypanosome (Mattioli and Wilson, 1996) since the former must undergo developmental changes in the later to complete its life cycle.

Intriguingly also, the defence system of the tsetse fly has been co-opted by the trypanosome and used as a tool to stimulate its own maturation in the fly as seen in the positive role of tsetse lectin in the maturation of midgut infections (Maudlin and Welburn, 1988a, Welburn and Maudlin, 1989). Inhibition of lectin activity prevented the maturation of the parasites beyond the procyclic stage but this was reversed following the restoration of lectin indicating that the process of trypanosome maturation is dependent upon a signal from the fly and not predetermined by a fixed number of division cycles by the parasites (Welburn and Maudlin, 1989). Also some tsetse salivary gland proteins are thought to be intimately associated with the maturation and development of trypanosomes in the salivary glands (Haddow et al., 2002). For instance, the expression of tsetse TSGF-1, a salivary protein with adenosine-deaminase activity is thought to allow survival of African trypanosomes in the salivary glands since these parasites are

known purine scavengers but lack adenosine deaminase, an enzyme involved in purine metabolism (Ogbunude et al., 1985). The development of *T. b. brucei* within the alimentary tract and salivary glands in *G. m. morsitans* are shown in Figure 1.10.

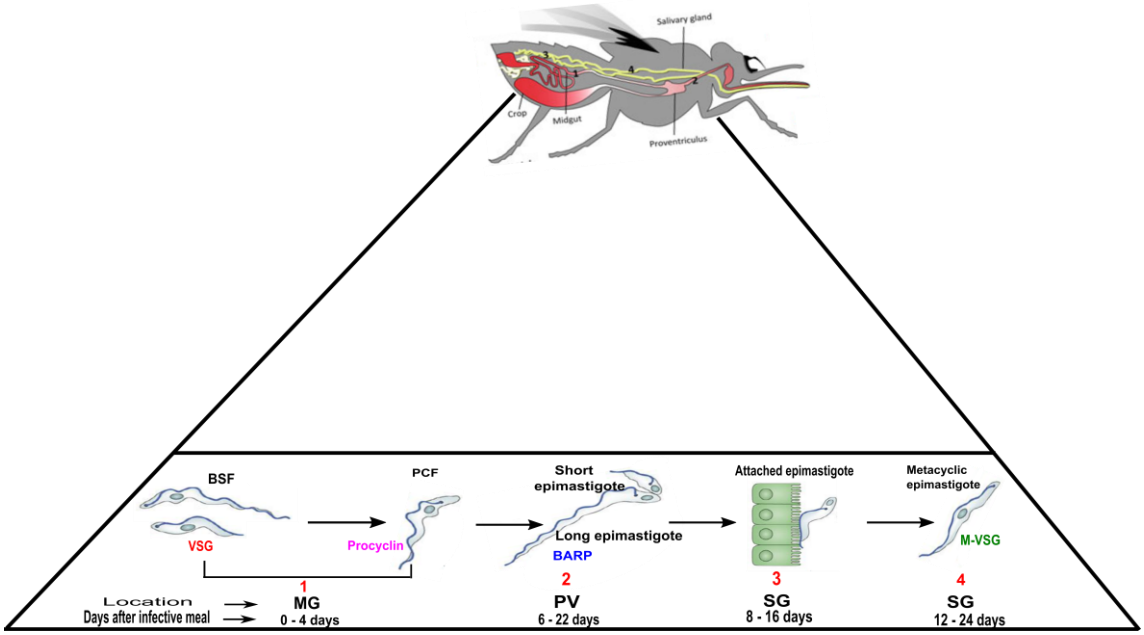

Figure 1.10 The spatio-temporal course of *T. b. brucei* infection in *G. m. morsitans*. The major sites of the major developmental pathway are shown. Following an infective bloodmeal, bloodstream forms (BSFs) taken by the fly rapidly differentiate to procyclics in the fly midgut (MG). The parasites migrate anteriorly to the proventriculus (PV) at 6 days transforming to long trypomastigotes which divide asymmetrically producing long and short epimastigotes. Colonization of the salivary glands occurs from day 8 with attached epimastigotes and develops into infective, metacyclics from day 12. The completion of this developmental cycle takes approximately 3 weeks (Adapted from (Langousis and Hill, 2014, Caljon et al., 2014).

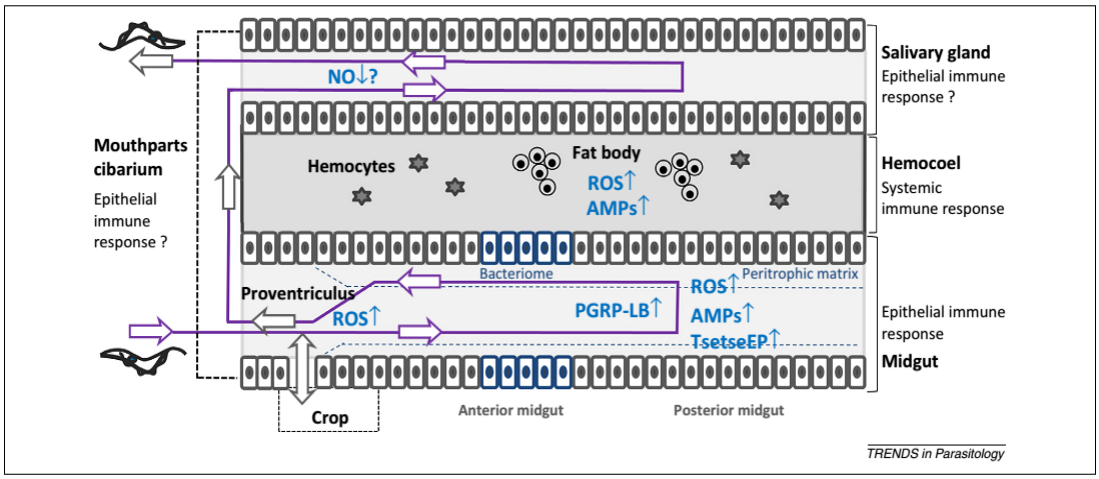

Figure 1.10 Overview of tsetse fly immune responses to trypanosome infection. Trypanosomes are ingested by the tsetse fly through blood meal on an infected host. Once inside the gut, the parasites have to go through a series of complex developmental changes in different compartments of the alimentary tract with the final infective metacyclic stage in the mouthparts (*T. congolense*) or salivary glands (*T. brucei*) (indicated by the purple line and arrows). The parasite elicits both local epithelial as well as systemic defence reactions in the fly, resulting in several effector molecules including reactive oxygen species (ROS), immune deficiency (IMD)-regulated antimicrobial peptides (AMPs) and the PGRP-LB scavenger receptor. The PGRP-LB level in the adult's midgut seems to be regulated by the obligatory bacteriome (*Wigglesworthia*) during larval development. The latter also affects the integrity of the midgut peritrophic matrix of the emerging adult fly. The peritrophic matrix is suggested to regulate the fly's ability to immunologically detect and respond to the parasite (Beschin et al., 2014).

1.11 Tsetse microbiota

Insects harbour bacterial endosymbionts which play important physiological roles such as metabolism, maintenance of fecundity and immune system development (Douglas, 2011). Tsetse flies are known to harbour three endogenous symbionts, obligate mutualist *Wigglesworthia glossinidia*, commensal *Sodalis glossinidius* and parasitic *Wolbachia pipientis*, which influence reproduction, immunity and fly fitness (Attardo et al., 2008, Pais et al., 2008, Alam et al., 2011). Both *Wigglesworthia* and *Sodalis* belong to the Enterobacteriaceae family and *Wigglesworthia* is made up of two distinct populations, one in the bacteriome that surrounds the anterior midgut of the tsetse fly (Aksoy, 1995, Aksoy, 2000),

and another found extracellularly in milk gland secretions (Attardo et al., 2008). *Sodalis*, however, exhibits a wide range of tissue tropism and is widespread in numerous tissues in the fly including midgut, fat body, milk gland, salivary glands and haemocoel (Cheng and Aksoy, 1999, Balmand et al., 2013). *Wolbachia* is found exclusively in germ line tissues and have been detected in early oocyte, embryo and larvae (Cheng et al., 2000, Balmand et al., 2013).

The presence of *Wigglesworthia* offers both nutritional and immunological benefits to tsetse. It supplements its tsetse host with nutrients such as vitamins that are either lacking or at low amounts in the vertebrate blood (Balmand et al., 2013). It has also been demonstrated that the presence of *Wigglesworthia* during intrauterine development is essential for proper immune functioning during the adult stage (Weiss et al., 2011, Weiss et al., 2012, Weiss et al., 2013, Wang et al., 2009). Unlike *Wigglesworthia*, several natural tsetse populations lack *Sodalis*, (Geiger et al., 2005, Lindh and Lehane, 2011) suggesting that the association between *Sodalis* and tsetse may be essentially commensal (Wang et al., 2013).

However, it has been shown that the presence of *Sodalis* increases the susceptibility of tsetse to trypanosome infection (Welburn et al., 1993) and the elimination of *Sodalis* through streptozotocin treatment results in tsetse being more resistant to trypanosome infection (Dale and Welburn, 2001). Also, there is a correlation between trypanosome infection prevalence and the presence of *Sodalis* (Farikou et al., 2010, Soumana et al., 2013). While the presence of *Wigglesworthia* increases tsetse refractoriness to trypanosome infection, it can be said that the presence of *Sodalis* favours trypanosome infection establishment in *Sodalis*-harbouring populations of tsetse. In tsetse, *Wolbachia* induces cytoplasmic incompatibility in which mating between *Wolbachia*-cured females and *Wolbachia*-infected males produces offspring that die early during embryogenesis (Alam et al., 2011). Both *Wigglesworthia* and *Sodalis* are transmitted via milk gland secretions while *Wolbachia* is transmitted transovarially via germ line cells.

1.12 Alteration of tsetse feeding behaviour by trypanosomes

In host-parasite interactions, alteration or modification of host behaviour by the parasite is designed to increase the chances of the parasites completing their life cycle and these modifications can include a change in preferred substrate, temperature preferences, locomotor activity, visual cycle, circadian rhythms, geo- or phototropism, cessation of food consumption, feeding rate and any other behaviours that may enhance the survival and transmission of the parasite (Adamo, 2002, Hurd, 2003, Lefevre and Thomas, 2008, Rogers and Bates, 2007, Schaub, 2006, Thomas et al., 2005, Webster, 2001). It has been demonstrated that in an arthropod vector-parasite association, the behaviours, particularly feeding behaviour, of the vector is modified by the parasite in order to increase the contact of the vector with the vertebrate host thereby maximising chances of transmission of the parasite to the vertebrate host (Schaub, 2006, Moore, 1993).

Tsetse flies infected with trypanosomes are known to exhibit increased probing rate and feed with more voracity than uninfected flies (Jenni et al., 1980, Roberts, 1981). However, the molecular mechanisms involved in these behavioural changes are not well understood (Biron et al., 2005, Libersat et al., 2009). There is evidence that trypanosomes can alter the head proteome of tsetse flies through modifications in energy metabolism, signal transduction and heat shock response (Lefevre et al., 2007) and this shows that a parasite has the ability to induce a global metabolism disorder that leads to nutritional stress and consequently to new feeding attempts (Lefevre and Thomas, 2008). It has also been suggested that trypanosomes can modulate the apoptosis pathways of tsetse during their interaction (Biron and Loxdale, 2013).

It has also been shown that the colonisation of the salivary glands of tsetse flies by trypanosomes leads to significant decrease in anti-haemostatic activities (anti-platelet aggregation and anti-thrombin activities) in addition to the inhibition of thrombin-induced blood coagulation; this leads to a decrease in the fly's feeding ability and makes it feed for longer periods and hence increases contact between vector and mammalian host thereby affording the parasites more opportunities for transmission (Van Den Abbeele et al., 2010).

A better understanding and characterisation of the molecular mechanisms involved in the cross-talk between trypanosomes and their tsetse fly vector which invariably leads to modifications in the behaviour of the vector that favours the transmission of the parasite to a vertebrate host will enable us have a better understanding of tsetse immune responses towards trypanosome infection. This will in turn enable us to design control strategies aimed at interfering with the transmission of African trypanosomiasis.

1.13 Aims and objectives

Trypanosomes have to overcome a number of natural bottlenecks in the tsetse fly vector to eventually reach the salivary glands or mouthparts of the fly where they differentiate into a final stage that is infective for a new vertebrate host. Based on a number of studies (Msangi et al., 1998, Otieno and Darji, 1979, Morlais et al., 1998, Auty et al., 2012, Jamonneau et al., 2004, Peacock et al., 2012b), it has been established that a high percentage of tsetse flies are resistant to trypanosome infection. The outcome of tsetse-trypanosome interactions is thought to be dependent on the efficiency of the innate immune response of the fly versus the ability of the parasites to suppress or evade the immune response of the fly.

In this thesis, I set out to characterise and functionally analyse several differentially expressed genes in the midgut of *G. m. morsitans* that were resistant (or self-cured) to *T. brucei* infection. A comprehensive bioinformatic analysis of immune related genes that were differentially expressed in the midgut of refractory flies, previously identified by 454 sequencing, is described in Chapter 3. I used RNAi silencing to validate the biological involvement of these genes in the susceptibility of the flies to trypanosome infection (Chapter 4). Among the four immune related candidate genes in refractory flies, I concentrated my efforts in characterising the role of a secreted PLA_2 activity. This enzyme has trypanocidal activity *in vitro* and its expression appears to be dependent on parasite density and time of infection in the fly. Surprisingly, $sPLA_2$ expression was found to be high in the midgut of flies infected by the gram-

positive bacterium, *S. aureus*, suggesting a possible involvement or modulation by the tsetse Toll pathway (Chapter 5).

The primary objective of this thesis was to investigate the interactions between tsetse and trypanosomes with a view to shed more light on the molecular mechanisms involved in tsetse-trypanosome crosstalk.

Chapter 2
General Materials and Methods

In this chapter, a general outline of the methods used in the course of this thesis is described, except for the bioinformatics methodology which is described in Chapter 3. References will be made as appropriate in subsequent chapters

2.1 Rearing and maintenance of tsetse

The *Glossina morsitans morsitans* colony was reared and maintained in an insectary at the Liverpool School of Tropical Medicine. The flies originally from Zimbabwe, were established in LSTM in 2002 from the University of Bristol colony. Flies were maintained at 26°C and 65 - 75% relative humidity and fed defibrinated horse blood (TCS Biosciences Ltd., Buckingham, UK) using an artificial feeding membrane (Moloo, 1971a). Male flies were used throughout the experiment.

2.2 Trypanosome stock

The strain of trypanosomes used in this experiment (kindly provided by Prof. Wendy Gibson, Bristol), *Trypanosoma brucei brucei* TSW196 (MSUS/CI/78/TSW 196) (CLONE A) was originally isolated in 1978 from a pig in Cote d'Ivoire (Mehlitz et al., 1982). Parasites were taken from rats during peak parasitaemia and blood stabilates were cryopreserved at -180°C until use.

2.3 Synthesis of dsRNA

dsRNA was synthesized with PCR products of the genes tailed with T7 RNA polymerase promoter sequence at their 5' ends using the MEGAscript High Yield T7 Transcription kit (Ambion, Huntington, UK) according to manufacturer's instructions. DNA plasmids from a tsetse midgut EST library (Lehane et al., 2003b) were used as templates while double stranded enhanced green

fluorescent protein (dsRNA-eGFP) was used as a negative control. The T7 primer sequences used are listed below.

GMOY000153 (chitinase)
Forward: TAATACGACTCACTATAGGGCCGCTGTTAAGCACAATCAA
Reverse: TAATACGACTCACTATAGGGCATTGATAGAAACGCGAGCA

GMOY002400 (OGT)
Forward: TAATACGACTCACTATAGGGCGAAGTCAGAGGGTTTCTGG
Reverse: TAATACGACTCACTATAGGGATACCGCAGCCACGTATTTC

GMOY009713 (PLA_2)
Forward: TAATACGACTCACTATAGGGATACGCTTGCACATTTACGAGAT
Reverse: TAATACGACTCACTATAGGGTGGCTGCGGCTTTACAACT

GMOY006016 (SPI)
Forward: TAATACGACTCACTATAGGGCGGCAAATAGTGATGATCCA
Reverse: TAATACGACTCACTATAGGGCTTGCAAGTTTCCTCGCATT

GFP
Forward: TAATACGACTCACTATAGGGACGTAAACGGCCACAAGTTC
Reverse: TAATACGACTCACTATAGGGCTTGTACAGCTCGTCCATGCC

The following PCR cycling conditions were used: 95°C for 5 minutes, 30 cycles of 95˚C for 1 minute, 55˚C for 30 seconds and 72˚C for 1 minute and a final extension of 72˚C for 5 minutes. Purification of dsRNA was done using MEGAclear™ columns (Ambion, Huntington, UK) and finally eluted in nuclease free water. The concentration of the synthesized dsRNA was measured using NanoVue™ Plus Spectrophotometer (GE Healthcare, Buckinghamshire, UK). Eluted dsRNA was concentrated to obtain a concentration of 5 µg/µl using DNA-Mini vacuum concentrator (Fisher Scientific, Loughborough, UK).

2.4 RNAi experimental design

In order to ascertain the roles of our genes of interest in controlling trypanosome infection in tsetse, we carried out RNAi-mediated knockdown of the genes of interest followed by quantitative (qPCR) analysis to confirm and validate their roles in trypanosome challenged flies. Teneral flies were fed defibrinated horse

blood on the day of emergence and those that did not take a blood meal were discarded. Flies were injected with 10 µg/fly dsRNA of gene of interest and dsRNA-GFP the following day, fed normal blood meal 24 hours after injection and allowed to rest for 24 hours. Flies that did not take a blood meal were discarded. The flies were then fed infectious blood meal containing *T. b. brucei* TSW196 bloodstream form parasites on day 5. Again flies that did not take a blood meal were discarded. The flies were subsequently maintained every 48 hours on defibrinated horse blood and dissected 7 days after infection to check for midgut infection (Figure 2.1).

2.4.1 Timing of Infectious blood meal

The infectious blood meal was supplied at the third feed. In the field tsetse flies are known to be resistant to trypanosome infection. Even in the laboratory where flies are made to take infected blood meal under experimental conditions, the proportion of flies that establish midgut infections are still small especially if the flies are made to take multiple blood meals prior to the infectious blood meal (Distelmans et al., 1982, Kubi et al., 2006). Flies were therefore fed infectious blood meal at the third blood meal in order to reproduce the refractory phenotype (Figure 2.1).

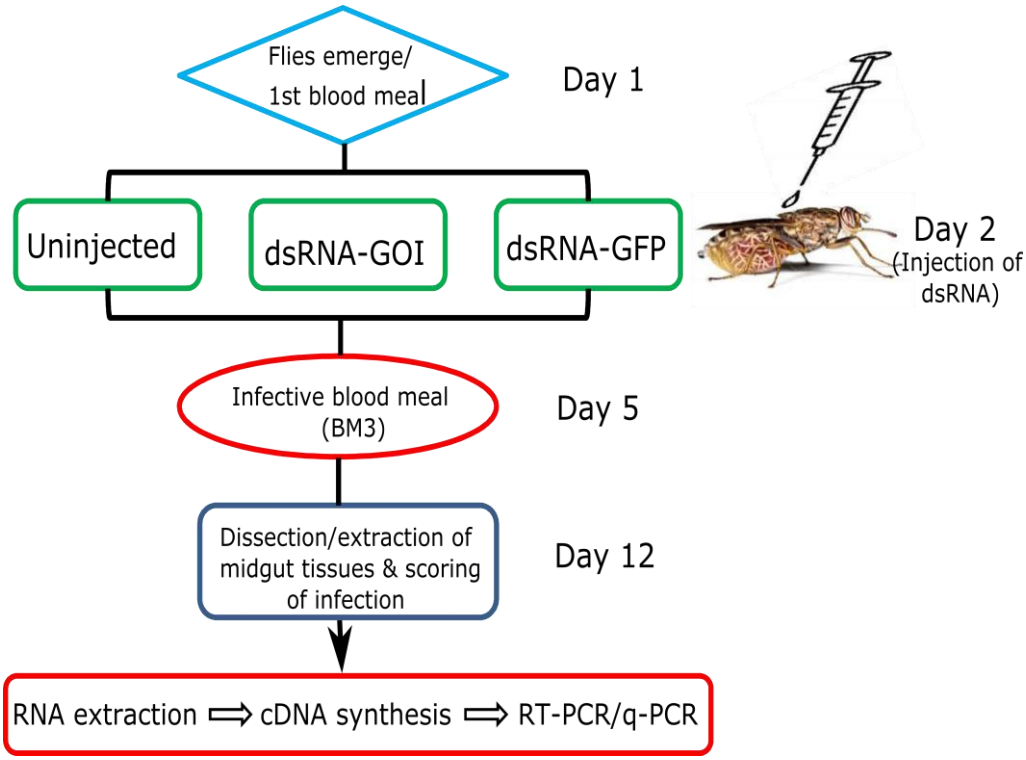

Figure 2.1 RNAi experimental design. Flies were fed with sterile horse blood on the day of emergence. The flies were injected with dsRNA of the gene of interest (dsRNA-GOI) on day 2. Flies were fed with sterile horse blood 24 hrs after injection (day 3) and infected with bloodstream form parasites on day 5. They were maintained on sterile horse blood every 48 hrs and dissected 7 days post-infection to check for midgut infection. Midgut tissues were collected for qPCR (transcript) and western blot (protein) analysis. Uninjected flies and dsRNA-GFP-injected flies were used as controls.

2.5 Injection dsRNA

The needles for injection were made in the lab using micro-haematocrit glass capillary tubes (2.00 mm outside diameter) (Globe Scientific, New Jersey, USA). The tubes were pulled to an approximate external tip diameter of 45 µl after heating using a needle puller (PC10; Narishige, Japan). Needles were checked to ensure there were no trapped glass particles inside before they were used for injection. Flies were injected 24 hours after receiving a blood meal. Flies were chilled on ice for 10 minutes to immobilize them prior to injection with dsRNA and were injected with 2 µl (10 µg) of dsRNA per fly at the dorsolateral surface of the thorax (scutum) and allowed to rest for 24 hours before the next bloodmeal. The

needle was angled slightly at 45° to minimise the impact of the pressure to the organs. The flies were fed 24 hours after injection.

2.6 Trypanosome infection

The infective blood meal was prepared by mixing *T. b. brucei* TSW196 stabilate in sterile defibrinated horse blood to achieve a final concentration of approximately $5x10^5$ parasites/ml. The flies were fed with the infective blood meal using an artificial feeding membrane (Moloo, 1971a). Flies that did not feed on the infective blood meal were discarded.

2.7 Scoring of infection rates in dsRNA knockdown flies

Flies were dissected 7 days after infection and the midguts were analysed by light microscopy for the presence of trypanosomes. Midguts were shredded into a drop of phosphate buffered saline (PBS) on a glass slide. Infection prevalence was determined by searching 10 random fields by dark field microscopy (x40 magnification) for the presence of motile trypanosomes.

2.8 Bacterial infection

E. coli strain K12 RM148 and *S. aureus* strain SH1000 were grown in LB broth and incubated overnight at 37°C with shaking**.** Bacterial suspensions were prepared and adjusted to OD600=0.5 in LB broth. The infectious blood meal was prepared by mixing 100 µl of bacterial suspension with 1 ml of defibrinated horse blood and subsequently fed to flies. Flies that did not take the blood meal were discarded.

2.9 Optimising conditions prior to RNA extraction

Obtaining high quality RNA is very critical in any molecular technique involving the use RNA. RNA is far less stable than DNA and must therefore be treated with extra care to avoid degradation by both endogenous RNAse in tissue and

exogenous RNAse. Contamination must be avoided. In this project, nuclease-free water was used in all experiments involving the use of RNA since water is one of the common sources of microbial nucleases. Also surfaces and containers were thoroughly cleaned using RNaseZap® spray (Ambion, TX, USA) prior to the commencement of RNA extraction.

2.10 RNA extraction

RNA was isolated from midgut tissues from dissected flies using TRIzol (Invitrogen, Paisley, UK) reagent. Briefly, frozen samples were homogenized in 500 µl of TRIzol reagent in 1.5 ml tube using PELLET PESTLE® (Kimble Chase, Vineland, NJ) homogenizer. Homogenized samples were left to incubate for 5 minutes at room temperature after which 100 µl of chloroform was added and the tubes were shaken vigorously by hand for 15 seconds and allowed to incubate at room temperature for 5 minutes. After incubation, the samples were centrifuged at 12,000 g for 15 minutes at 4˚C. The upper aqueous phase was carefully transferred to a new tube and 250 µl of isopropanol was added and left to incubate at room temperature for 10 minutes. The mixture was then centrifuged at 12,000 g at 4˚C for 10 minutes and the supernatant was carefully removed from the pellet. The RNA pellet was washed with 1 ml 75% ethanol (made with RNAse free water) and supernatant was carefully removed and the RNA pellet was allowed to air dry until all the ethanol evaporated. The RNA pellet was then dissolved in 30 µl of nuclease free water by gently pipetting up and down. After extraction, samples were treated with DNAse to remove contaminating DNA. Briefly, 3 µl of 10X TURBO DNase Buffer and 1 µl of TURBO DNase was added to the RNA sample mixed gently. The mixture was incubated at 37 ˚C for 30 minutes. Then 3 µl of resuspended inactivation buffer was added and the mixture was incubated at room temperature for 5 minutes with intermittent mixing (3 times) to redisperse the DNase Inactivation reagent. Finally, the mixture was centrifuged at 10,000 x g for 1.5 minutes and the RNA was transferred to a fresh 1.5 ml tube and stored at -80 ˚C.

2.11 RNA analysis

Following RNA extraction, a NanoVue™ PLus (GE Healthcare, Buckinghamshire, UK) spectrophotometer was used to quantify the yield and purity by measuring its absorbance at 260 nm (A_{260}). RNA samples that were stored at -80°C were thawed on ice prior to measurements. The sample measurement pedestal was cleaned with a dry lint-free laboratory wipe before initialising it with a blank (2 µl nuclease free water used to dissolve the RNA). After this the sample pedestal was cleaned as previously described and 2 µl of each sample was put on the sample pedestal and measured. The ratio of absorbance at 260 nm versus 280 nm (A_{260}/A_{280}) was used to assess the level of protein contamination, while the ratio of 260 nm versus 230 nm (A_{260}/A_{230}) was to assess the level of contamination by organic compounds. The values of the A_{260}/A_{280} and A_{260}/A_{230} ratios of the samples used in these studies were between 1.6 to 2.2 and 1.5 to 1.8 respectively. These values indicate the absence of significant amounts of contaminants and therefore meets the requirements for good quality RNA (Green and Sambrook, 2012).

2.12 cDNA synthesis

The quantified RNA was used to generate cDNA for amplification using polymerase chain reaction (PCR). The reverse transcriptase method using oligo-dT primers was used for cDNA synthesis. A sample of 1 µg of RNA was diluted in 8 µl of nuclease free water in 0.2 ml PCR tubes and the following reagents were added to bring the volume to 13 µl: 1 µl of dNTP mix (10 mM each) and 3 µl of nuclease free water. The mixture was heated at 65°C for 5 minutes in a PCR machine to denature the RNA and thus enable the adhesion of the oligo-dT primer onto the denatured RNA. Following this, the tubes were chilled on ice for 1 minute to enable the primers to anneal. After spinning down to collect the contents of the tube, the reverse transcription (RT) mix was added in the following order: 4 µl First-strand buffer 5X; 1 µl 0.1 M dithiothreitol; 1 µl RNAseOUT™ (40 U/µl) and 1 µl SuperScript III RT™ (200 U/µl). The mixture was then incubated at 50°C for 60 minutes in a PCR machine followed by 70°C

for 15 minutes to inactivate the reaction. The resultant cDNA was then stored at -20°C until use.

2.13 Primer design for PCR reaction

To design oligonucleotide sequences specific to the DNA of the genes of interest, Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/), a web-based free software for primer design was used. The first step was downloading the sequence for each gene from VectorBase. The sequences were pasted on the Primer3Plus site adjusting few of the parameters. The “Min” and “Max” of the “Primer GC%” was changed to 40.0 and 60.0 respectively. The product size range was set at 100-200 for qPCR primers and 450-650 for dsRNA primers while the “SantaLucia 1998” method was chosen for both “Table of thermodynamic parameters” and “Salt correction formula”. To minimise hairpin formation, “Max Self Complementarity” and “Max 3′ Self Complementarity” was tuned down. The rest of the parameters were left at default setting. After setting all parameters, the “Pick Primers” option was chosen. This generates a list of primer pairs that meet all of the input requirements. The primer pairs chosen had similar melting temperatures (differences of ± 1°C). Primers were checked for potential off-target binding using BLASTn search while relaxing the *E*-value threshold to 200 to detect more potential off-targets. α-tubulin and β-tubulin were the housekeeping genes used to normalise for the qPCR experiments described in subsequent chapters. Primer sequences for β-*tubulin* were taken from (Weiss et al., 2012). Table 2.1 shows the list of primers used.

Table 2.1 List of primer sets used in this thesis

Procedure	Gene	Primer sets	Product Size (bp)	Tm (˚C)
dsRNA	GMOY000153 (Chitinase)	F-5'-TAATACGACTCACTATAGGGCCGCTGTTAAGCACAATCAA-3' R-5'-TAATACGACTCACTATAGGGCATTGATAGAAACGCGAGCA-3'	472	59
	GMOY002400 (OGT)	F-5'-TAATACGACTCACTATAGGGCGAAGTCAGAGGGTTTCTGG-3' R-5'-TAATACGACTCACTATAGGGATACCGCAGCCACGTATTTC-3'	447	60
	GMOY009713 (PLA_2)	F-5'-TAATACGACTCACTATAGGGATACGCTTGCACATTTACGAGAT-3' R-5'-TAATACGACTCACTATAGGGTGGCTGCGGCTTTACAACT-3'	444	58
	GMOY006016 (SPI)	F-5'-TAATACGACTCACTATAGGGCGGCAAATAGTGATGATCCA-3' R-5'-TAATACGACTCACTATAGGGCTTGCAAGTTTCCTCGCATT-3'	441	60
	GFP	F-5'-TAATACGACTCACTATAGGGACGTAAACGGCCACAAGTTC-3' R-5'-TAATACGACTCACTATAGGGCTTGTACAGCTCGTCCATGCC-3'	636	60
qPCR	GMOY000153 (Chitinase)	F-5'-AAATTCACACACGCGGCAAA-3' R-5'-TGCCGAGTGTTGAAAATTGCA-3'	155	55
	GMOY002400 (OGT)	F-5'-TATAGGAGTCTCGCCGGAAC-3' R-5'-TAAGTTCGGGACAACCAAGG-3'	152	56
	GMOY009713 (PLA_2)	F-5'-TTCACCGGCACCCTAACTTT-3' R-5'-ATCCCTTTGTCGATTCCCAGCA-3'	158	58
	GMOY006016 (SPI)	F-5'-CCGCTGTTAAGCACAATCAA-3' R-5'-CATTGATAGAAACGCGAGCA-3'	198	57
	α-tubulin	F-5'-TGT ATG TTG TAT CGT GGT GAT GT-3' R-5'-GAA TTG GAT GGT GCG TTT AGT TT-3'	146	55
	β-tubulin	F-5'-CCATTCCCACGTCTTCACTT -3' R-5'-GACCATGACGTGGATCACAG -3'	152	55

2.14 Agarose gel electrophoresis

Agarose gels were prepared to separate and analyse the amplified DNA (PCR products) of the genes of interest. A 1% agarose gel was prepared by mixing 0.5 g multipurpose molecular grade agarose in 50 ml of Tris-Acetate-EDTA (TAE) buffer in a 250 ml flask. This was heated to melt the agarose and swirled to ensure even mixing and allowed to cool to about 55˚C. Then 5 µl of SYBR Safe (Invitrogen, UK) was added and swirled to get a good mix. The liquefied solution was then poured into a Multi Sub Mini 7 x 10cm horizontal agarose Gel Tray (Geneflow, Lichfield, UK) that holds 2 8-tooth combs and sealed with rubber tray dams at the ends. The gel was left to set at room temperature for 30 minutes and then transferred into a gel electrophoresis tank (Geneflow, Lichfield, UK) containing TAE buffer which was topped up to ensure the gel is submerged in TAE buffer. The combs were then removed carefully and 5 µl of amplified DNA

(PCR product) was loaded into each well. The electrophoresis tank was then covered with a lid before connecting it to a power source through a gel electrophoresis power pack (Consort BVBA, Turnhout, Belgium). The gel was run at 90V for 45 minutes.

2.15 Gel imaging

The gel was taken out of the electrophoresis tank at the end of the run and drained of excess TAE buffer. This was then placed in a tray for nucleic acid applications with SYBR stains and then placed in the Bio-Rad Gel Doc EZ imager (Bio-Rad Laboratories, UK) to view and capture the gel image. The captured image was then saved in TIF format.

2.16 Quantitative polymerase chain reaction (QPCR)

The methodology used to measure gene expression in the quantitative polymerase chain reaction in these experiments is the Brilliant III Ultra-Fast SYBR® Green dye detection system by Agilent Technologies. This method uses the fluorogenic, highly specific, double-stranded DNA-binding dye, SYBR Green I to detect the PCR product as it accumulates during the PCR reaction. The dye is added at the start of the reaction and binds to all double-stranded DNA present resulting in the emission of a strong fluorescent signal. Because many dye molecules can bind to each DNA product and since the target sequence is amplified as the PCR cycle progresses, the intensity of signal generated is high and proportional to the total mass of DNA generated during the PCR reaction. Primer pairs for genes of interest were designed and optimised using Primer3Plus (see section 2.10) for use with SYBR Green I assay.

2.16.1 QPCR workflow

Following the synthesis of cDNA from isolated RNA, a serial dilution of the cDNA with nuclease-free water was carried out to determine the efficiency of the

qPRC. An appropriate volume of PCR master mix (10 µl) containing SYBR Green dye, buffer, thermostable hot-start recombinant Taq DNA Polymerase and deoxynucleotides (dNTPs) was added to 3 µl of nuclease-free water. A separate mixture of 1 µl of each primer stock solution and 5 µl of template cDNA was also prepared. Both master mix and stock mix were mixed together in the appropriate reaction tubes (0.2 ml strip-capped tubes by pipetting to give a final reaction volume of 20 µl. A separate no-template reaction mixture was also set up as control. All samples were prepared in triplicate. The tubes were then placed in an MxPro – Mx3005P thermocycler (Agilent Technologies) and run after setting the programme using the following thermal cycling parameters: 95 ˚C for 10 minutes, followed by 40 cycles of 95 ˚C for 15 seconds, 60 ˚C for 1 minute and then one cycle of 95 ˚C for 1 minute, 55 ˚C for 30seconds, 95 ˚C for 30 seconds.

2.16.2 QPCR data analysis

The experimental procedures outlined above were aimed at the determination of fold changes in the expression levels of the genes of interest. The fold change in the expression levels of all genes of interest relative to control was calculated using the comparative C_T method (the $\Delta\Delta C_T$ method) (Livak and Schmittgen, 2001). The threshold cycle (CT) values (a threshold for detection of DNA-based fluorescence when the level of fluorescence gives signal above the background) were obtained. These C_T values were normalised to two housekeeping genes to give the ΔC_T value by subtracting the geometric mean of the housekeeping genes C_T value from the average C_T value of the gene of interest (ΔC_T = C_T gene of interest – C_T housekeeping gene). The $\Delta\Delta C_T$ value was then calculated by subtracting the ΔC_T of the untreated sample (control) from the ΔC_T of the gene of interest ($\Delta\Delta C_T$ = ΔC_T gene of interest - ΔC_T untreated control). This value was then substituted into the equation $2^{-\Delta\Delta Ct}$ (2= fold change in PCR products between cycles, $\Delta\Delta C_T$ = normalised cycle changes between gene of interest and untreated control), to get the fold change. CT values were averaged across triplicates and delta CT values were calculated between each test gene and control.

2.16.3 Reference genes

Reference or housekeeping genes are used to normalise data in qPCR experiments to eliminate, as much as possible, errors arising from biological variation of samples (Vandesompele et al. 2009) or experimental errors introduced as a result of variation in instrumentation or pipetting techniques (Dheda, Huggett et al. 2005). A normalisation strategy based on the use of a single housekeeping gene leads to relatively large errors while the use of multiple validated stably expressed housekeeping genes for normalisation gives a more accurate result (Vandesompele et al. 2002). For this reason, two housekeeping genes, *α*-tubulin and *β*-tubulin were used as reference genes to normalise for gene expression.

2.16.4 Sensitivity and specificity of primer sets for QPCR

Primer sets used in QPCR reactions were tested in PCR assays against cDNA samples. Ten-fold dilution series of cDNA samples were used to test the efficiency of PCR amplification used for each primer set. Individual PCR assays were performed at each dilution using a reaction volume of 20 µl (3 µl of nuclease-free water, 10 µl of PCR master mix, 1 µl each of forward and reverse primers and 5 µl of cDNA). Standard curves were generated for each of the tested primers to determine the efficiency of the PCR set up.

2.17 Confirmation of gene knockdown and susceptibility to trypanosome infection after gene knockdown.

Flies were fed normal blood meal on the day of emergence (<24 hours post-eclosion) and unfed flies were discarded. Each fly was then injected with 10 µg of dsRNA of genes of interest on the second day. A set of flies were also injected with dsRNA-GFP as internal control and another set were left uninjected. All flies were then fed trypanosome infected blood meal (*T. B. brucei* BSFs) 24 hours after injection. Again flies that did not take infective blood meal were discarded. Flies were dissected 7 days after infection, scored for trypanosome infection and

midgut tissues extracted. Midguts were shredded into 50 µl of phosphate buffered saline (PBS) on a clean glass slide and observed under light microscopy (x40 magnification) to search for motile trypanosomes and establish the presence of infection. Extracted midgut tissues were collected in 1.5 ml Eppendorf tubes and snap-frozen in liquid nitrogen, and later stored at -80 °C until QPCR analysis.

2.18 SDS-PAGE and Western blot analysis

Western blot analysis is a combination of electrophoretic separation of proteins and immunological detection, since proteins are separated by size electrophoretically and then detected immunologically by specifically directed antibodies. The technique essentially confirms the presence of a target protein in any given sample as well as compares the relative levels of protein expression between samples.

2.18.1 Separation of proteins by gel electrophoresis

To separate proteins, the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is commonly used. In this method, an electrical field is applied which moves through a gel matrix. SDS-PAGE separates proteins according to molecular size, just as horizontal agarose gel electrophoresis separates DNA molecules, but because DNA molecules are negatively charged, they will be pulled toward the positive end of the gel. Proteins, however, are not negatively charged and their secondary tertiary structures must be overcome if proteins are to be accurately separated based on size. Therefore the treatment of proteins with a detergent such as SDS, is to make them unfold into a linear shape as well as coating the proteins with a net negative charge, which allows them to migrate toward the positive end of the gel and be separated when an electrical field is applied. The incorporation of a reducing agent such as dithiothreitol (DTT) in the gel helps in breaking the disulphide bonds holding the tertiary structure together. Also heating the protein sample helps in the

denaturation and unfolding process allowing chemicals such as SDS and DTT to interact with the protein. The SDS-coated protein molecules move through the pores of the gel matrix in the presence of an electrical field, with smaller molecular weight proteins moving more quickly than larger ones. The proteins in the sample are therefore separated according to their relative molecular weight and size.

2.18.2 Detection of bound antibody

After separation, the proteins are transferred from the gel onto a protein-binding membrane. All of the protein bands that were originally on the gel are transferred onto the membrane at the end of the transfer process. Following the transfer of proteins onto a membrane, the membrane is blocked to prevent the occurrence of any non-specific reaction. After this the membrane is incubated with a primary antibody that specifically binds to the target protein. Any unbound antibody is washed away at the end of the incubation leaving only the bound antibody to the protein of interest. The membrane is then incubated with another (secondary) antibody that is able to recognise and bind to the first antibody. The secondary antibody is conjugated with an enzyme that produces colour or light which can be detected.

2.19 Timecourse analysis of PLA_2 expression in the midgut after trypanosome and bacterial infection

Time course experiments were carried out to monitor the expression of PLA_2 in the midgut following trypanosome and bacterial challenge as described below.

2.19.1 Timecourse analysis of PLA_2 expression after infection with BSF trypanosomes

To monitor the expression of $sPLA_2$ in the midgut of tsetse over the course of trypanosome infection, a time course experiment using midguts taken at different time points (0, 5, 10, 14 and 15 days post-infection) was performed to measure

PLA_2 transcript expression. Flies were dissected to collect midgut tissues from infected flies at the time points stated above, snap-frozen in liquid nitrogen and subsequently stored at -80 °C. These samples were later analysed for PLA_2 transcript expression using qPCR.

2.19.2 Timecourse analysis of $sPLA_2$ expression after infection with PCF trypanosomes

In order to test if the expression of $sPLA_2$ in the midgut of the fly is influenced by the differentiation of the parasites into procyclic stage, flies were infected with *T. b. brucei* PCFs. The infectious bloodmeal was prepared as follows: defibrinated horse blood (TCS Biologicals, Buckingham, UK) was centrifuged at 2000 *rpm* for 10 minutes, and pelleted (RBCs) were washed three times in complete SDM-79 medium without antibiotics. Procyclic forms of TSW 196 were grown for one passage in SDM-79 medium without antibiotics. This was centrifuged at 1500 rpm for 5 minutes to collect the cells. The cells were washed three times in SDM-79 containing 10% FBS without antibiotics. The washed red blood cells were reconstituted to 5 ml with SDM-79 medium containing 10% FBS plus PCF TSW196. Two concentrations (1×10^5 ml^{-1} and 1×10^6 ml^{-1}) were prepared. Teneral flies were then infected by artificial feeding on a silicone membrane. Flies were dissected to collect midgut tissues at the same time points as above and analysed for $sPLA_2$ transcript expression as previously stated (see section 2.16.1).

2.19.3 Timecourse analysis of $sPLA_2$ expression after infection with heat-killed PCFs

To check if the expression of $sPLA_2$ is triggered just by the presence of trypanosomes, flies were challenged with blood meal containing heat-killed procyclic forms of TSW196. Procyclic forms trypanosomes were inactivated by heat treatment. The log phase culture in SDM79 10% FBS was heated at 58 °C for 5 minutes. Cells were checked for viability to ensure that the cells were all dead by staining 100 µl of heat-killed procyclics with 3mM methylene blue (final concentration) for 15 minutes at room temperature, in the dark. Then 1mL of

SDM79 was added and centrifuged at 2000 rpm for 5 minutes to pellet the cells. The cells were then re-suspended in 200 µl of SDM79 and later added to 5 ml washed horse RBCs to give a final concentration of 1 x 10^7 ml^{-1} and fed to teneral flies by artificial feeding on a silicone membrane. Flies were dissected to collect midgut tissues at the same time points and analysed for $sPLA_2$ transcript expression as previously stated (see section 2.16.1).

2.19.4 Timecourse analysis of $sPLA_2$ expression after challenge with bacteria

E. coli strain K12 RM148 and *S. aureus* strain SH1000 were grown in LB broth and incubated overnight at 37°C with shaking. Bacterial suspensions were prepared and adjusted to OD600=0.5 in LB broth. The infectious blood meal was prepared by mixing 100 µl of bacterial suspension with 1 ml of defibrinated horse blood and subsequently fed to flies. Flies that did not take the blood meal were discarded. Flies were dissected at the same time points as stated in section 2.16.1 to extract midgut tissues for analysis of $sPLA_2$ transcript expression as previously stated (see section 2.16.1).

2.19.5 Timecourse analysis of PLA_2 expression after challenge with heat-killed bacteria

To see if the expression of $sPLA_2$ can be triggered by challenging tsetse with dead bacteria, flies were challenged. Bacterial suspensions were prepared to an OD600=0.5. Heat-killed bacteria were prepared by incubating *E. coli* cells at 75°C for 10 minutes and *S. aureus* cells at 100°C for 35 min. Bacterial death was confirmed by inoculating 5 ml LB liquid culture and streaking 200 µl of each heat-killed *E. coli* and *S. aureus* onto LB plates and incubated overnight at 37 °C. These produced no growth after 24 hours. The heat-killed bacteria (500 µl) were diluted in 450 µl defibrinated horse blood which was subsequently fed to flies. Flies that did not take the blood meal were discarded. Flies were again dissected at the same time points as stated in section 2.16.1 to extract midgut tissues for analysis of PLA_2 transcript expression as previously stated (see section 2.16.1).

2.20 Western blot analysis of $sPLA_2$ expression after gene knockdown and at different time points after infection of tsetse with T. b. brucei

12.5% polyacrylamide resolving mini gel (40% (w/v) Acrylamide/Bis-acrylamide; 1.5 M Tris-Cl; 0.1% (w/v) SDS; 0.05% (w/v) ammonium persulfate (APS); 0.5% (v/v) tetramethyleneethylenediamine (TEMED)) was cast at 1.50 mm thickness and allowed to set. Once set, the resolving gel was topped with 3% stacking gel (40% (w/v) Acrylamide/Bis-acrylamide; 0.5 M Tris-Cl; 1% (w/v) SDS; 0.1% (w/v) APS; 0.08% (v/v) TEMED). Protein samples from midgut tissues were used for western blot analysis. Samples were mixed with an equal volume of Laemmli buffer (0.5 M Tris-Cl, 4% SDS, 20% glycerol, 0.2% bromophenol blue, 3% DTT), boiled at 95°C for ten minutes, then samples and molecular weight markers (prestained protein marker, broad range (6-175 kDa); New England Biolabs, Herts, UK) were loaded on 12.5% SDS polyacrylamide gel, then run at 200 V for 45 minutes in running buffer (0.025 M Tris, 0.192 M Glycine, 0.1% SDS pH 8.3) at room temperature. Proteins were transferred to Hybond^{TM}-P polyvinylidene difluoride (PVDF) transfer membrane (Amersham Biosciences, Ersham UK) and soaked in blocking buffer (5% skimmed dry milk in Tris Buffered Saline (TBS) buffer (pH of 7.4 and 0.01 M Tris Base, 153 mM NaCl)) for 1 hour.

Membrane was incubated overnight at 4°C in anti-$sPLA_2$ goat polyclonal antibody (Santa Cruz Biotechnology) diluted 1:200 in TBST (TBS with 1% Tween 20 and 2% skimmed dry milk). The following the day, membrane was washed in TBST and then incubated for 2 hours with gentle shaking at room temperature in 1:50000 mouse anti-goat IgG HRPO (Santa z Biotechnology, Santa Cruz, CA). Membrane was washed in TBST and HRPO chemiluminescent substrate (Super signal West Dura peroxide buffer and Super signal West Dura Luminol/ Enhancer solution) (Thermo Fisher Scientific Inc. Rockford, IL) was subsequently added. Kodak Biomax MR Film (Eastman Kodak Company, Rochester, NY) was used for chemiluminescence detection. PVDF membrane was stained with 0.2% (w/v) nigrosine. The developed film was superimposed on the stained PVDF membrane to indicate the precise location of protein bands and to establish that equal loading of protein per lane was achieved.

2.21 Expression and purification of G. m. morsitans recombinant sPLA$_2$ protein

Both the expression and purification of *G. m. morsitans* PLA$_2$ were done in collaboration with Dr. Martin J. Boulanger (University of Victoria, Canada). Briefly, a clone encoding *Glossina morsitans* sPLA2 (aa 4-173) was generated in a modified pAcGP67 vector incorporating an N-terminal hexahistidine tag and TEV cleavage site. To generate PLA2 (4-173) encoding virus for insect cell protein production, the PLA2 clone was transfected with linearized baculovirus into Sf-9 cells as follows: 2ml of Sf-9 cells at 0.46 x 10^6 cells/ml were allowed to adhere to the surface of a 6-well tissue culture plate for ~30minutes to form a confluent monolayer. Expression was performed by transfecting Hi-5 cells for 48 hours, after which time the supernatant was harvested, concentrated, and buffer exchanged into Binding buffer (50 mM Tris-HCL, pH 8.0, 200 mM $CaCl_2$, 200 mM KCl, 30 mM imidazole, 0.05% triton x-100). The expressed protein was affinity purified using 2.0 ml nickel beads. Protein was eluted using elution buffer (50 mM Tris-HCL, pH8, 200 mM $CaCl_2$, 200 mM KCl, 250 mM imidazole, 0.05% triton X-100), analyzed by SDS-PAGE, pooled and concentrated. From 3.2 L of cells, 1.33 mg of protein was purified.

2.22 Measurement of *G. morsitans* rec-sPLA$_2$ activity

PLA$_2$s are soluble in water, but their activity is greatly enhanced in lipid interfaces. Radiometric methods are mostly used for the measurement of PLA$_2$ activity because of their high sensitivity and selectivity, but they are laborious, expensive, involves radiochemical hazards and often require chromatographic purification of the products (Kitsiouli et al., 1999). The activity of rec-sPLA$_2$ was measured by a fluorimetric method (Kitsiouli et al., 1999) using the fluorescent phospholipid 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine (C_{12}-NBD-PC) (Avanti Polar Lipids, Alabaster, AL) as the substrate in the presence of bovine serum albumin (BSA) (Kitsiouli et al., 1999). Briefly, the fluorimetric phospholipid was dissolved in chloroform to prepare 20 µM stock solution and 10% bovine serum albumin was prepared in sterilized distilled water. The reaction mixture (1 ml final volume) was prepared by sequentially adding 50 mM Tris-HCL buffer (pH 8.0), 20 mM $CaCl_2$ and enzyme extract. The reaction was initiated by addition of 20 µM C_{12}-NBD-PC substrate and subsequently fluorescence intensity was continuously

monitored with Varioskan™ Flash Multimode Reader (Thermo Scientific™, Waltham, MA). Excitation and emission wavelengths were adjusted to 460 nm and 534 nm, respectively. The specific enzyme activity was calculated as described (Radvanyi et al., 1989). Commercial bee venom PLA_2 was used as reference enzyme. These assays were done at LSTM in collaboration with Lívia Cardoso, a visitor from Federal University of Rio de Janeiro, Brazil.

2.23 $sPLA_2$ killing Assay

To determine if *Gm*PLA_2 has trypanocidal activity, a Minimum Inhibitory Concentration (MIC) assay was performed on trypanosomes using the chromogenic/fluorogenic substrate alarmarBlue (Onyango et al., 2000), as previously described (Haines et al., 2003), but slightly modified. Briefly, healthy log phase PCF trypanosomes in log phase were adjusted to $2.0x10^4$ cells/mL in fresh medium and 100 µl were seeded into wells of a 96-well polypropylene microtitre plates with rounded bottom (COSTAR/Corning Inc, Corning, New York) already containing serial dilutions of the recombinant *G. m. morsitans* $sPLA_2$. Incubation was performed at 27°C for 66 hours after which 10 µl of alamarBlue (BioSource International, Inc, Camarillo, CA) was added to each well and the plate was incubated for an additional 6 hours making a final incubation period of 72 hours. At the end of the final incubation, 70 µl of cell free supernatant from each well were transferred into a 96-well flat-bottomed black/white microplate (Greiner Bio-One, CellStar, Brockville ON) for measurement of fluorescence using a Cytoflour 2300 microplate reader (Millipore, Bedford, MA). Excitation and emission wavelengths were adjusted to 540 nm and 590 nm respectively. The alamarBlue assay is a bioassay that combines both fluorimetric and colorimetric growth indicators based on detection of mitochondrial activity (Ahmed et al., 1994). The bioassay incorporates an oxidation-reduction indicator that responds to chemical reduction of a growth medium by metabolically active cells by way of fluorescence and colour change (Lancaster and Fields, 1997). A chemical reduction of alamarBlue occurs as a result of the metabolic activity of cells which causes the indicator to change from

oxidised (blue, non-fluorescent) to reduced (red, fluorescent). These assays were performed in collaboration with Lívia Cardoso.

2.24 Roche 454 sequencing data

Two groups of teneral flies were fed defibrinated horse blood on the day of emergence. Group 1 was maintained on defibrinated horse blood every two days while group 2 was infected with bloodstream form *T. b. brucei* TSW196 on the fourth blood meal. Both groups were dissected 3 days later and the midguts were carefully extracted and snap-frozen in an RNAse-free Eppendorf. Total RNA was extracted and quality assured using a bioanalyzer. A cDNA library for the two groups was prepared using SMARTer PCR and cDNA synthesis (Clontech, Mountain View, US), according to the manufacturer's instructions. Briefly, 1 µl of total RNA was added to 1 µl of 3′ SMART CDS Primer II A (12 µM) followed by mixing in2.4 µl of NFW with a pipette. The mixture was centrifuged briefly and incubated at 72˚C in a thermal cycler for 3 minutes and then at 42˚C for 2 minutes. The master mix was added in the following order: 2 µl of 5X First-Strand Buffer; 0.25 µl DTT (100 mM); 1 µl dNTP Mix (10 mM); 1 µl SMARTer II A Oligonucleotide (12 µM); 0.25 µl RNase Inhibitor; 1 µl SMARTScribe Reverse Transcriptase (100 U) (added just prior to use). This was incubated at 42°C for 1 hour followed by 10 minutes at 70˚C to inactivate the reaction.

The cDNA produced was sequenced using the 454 sequencing platform at the Genomics Suite, University of Liverpool. The transcriptome data comprised of reads from control (uninfected) flies and flies fed an infective bloodmeal containing *T. b. brucei* TSW196. This latter group contained a mixture of self-cured (refractory) and infected (susceptible) flies, however susceptibility to trypanosomes introduced at the 4^{th} bloodmeal is typically less than 5%. Consequently, this group is deemed naturally refractory to infection. To maintain high transcript integrity, the midguts were quickly excised and frozen and consequently, not screened for infection prevalence. We therefore refer to these two groups of flies as 'control flies (Group 1) vs trypanosome-challenged (Group 2)'.

2.25 Statistical analysis

Each experiment was repeated independently three times. Error bars represent the standard error of the mean of three replicates. Statistical significance of results was calculated using standard paired sample *t*-test and considered significant if p-values were lower than 0.05. Level of significance is indicated by asterisks, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

CHAPTER 3

Bioinformatic analysis of differentially expressed midgut genes in refractory *G. m. morsitans*

3.1 Introduction

Trypanosomes undergo a complex life cycle in the fly involving a series of morphological and biochemical changes that culminate in the development of mammalian infective metacyclics in the salivary glands (*T. brucei*) or the mouthparts (*T. congolense*). Once in the fly's midgut, the stumpy forms which are thought to be pre-adapted for life in the tsetse midgut differentiate into procyclics (Savage et al., 2012, MacGregor and Matthews, 2010). However, the majority of flies can effectively eliminate the parasites before they develop further (Aksoy et al., 2003). One would expect that as a result of the high disease burden in Africa, there would be a corresponding large number of flies that are also infected, yet paradoxically, just very few number of flies (<5%) can actually transmit the parasites to susceptible mammalian hosts (Aksoy et al., 2003). Even under ideal laboratory conditions, where each experimental fly takes an infected blood meal, the proportion of flies that harbour infective metacyclic trypanosomes are low (Rio et al., 2004, Aksoy et al., 2003). This means that tsetse flies are generally resistant to trypanosome infection, but the molecular mechanisms underlying this phenomenon are yet to be elucidated. Unravelling the molecular mechanism underlying tsetse-trypanosome interaction has remained elusive despite the immense amount of research associated with it.

A crucial step in the development of trypanosomes occurs when the parasites invade the fly midgut where they must survive in order to continue their developmental cycle. It follows therefore that for trypanosomes to have a chance of completing their life cycle in the fly they must first of all establish infection in the midgut of the fly. The life cycle of trypanosomes in the fly can be said to occur in two phases: firstly, the differentiation, colonisation, and proliferation in the midgut, and secondly, the migration, to the salivary glands or mouthparts (characterised by several rounds of differentiation) where the parasites mature into metacyclic forms which are capable of infecting new mammalian hosts

(Vassella et al., 2000). The molecular mechanisms underlying this intricate interaction are not fully understood. The tsetse midgut is of tremendous importance in the life cycle of trypanosomes, being the first port of call for the parasites after they are taken up by a tsetse fly while feeding on an infected mammalian host. Here the trypanosomes are met with a completely different type of environment, in addition to being exposed to the fly's digestive enzymes and potent immune molecules; to survive, the trypanosomes must respond to these threats to their continued survival in the fly's midgut and they do so by undergoing series of remarkable biochemical and physiological changes (Aksoy et al., 2003). As a result, a molecular cross-talk ensues between the tsetse fly and trypanosomes and the parasites can either be stopped in their tracks in the midgut or they survive and continue their developmental cycle. So as the fly tries to eliminate the parasites, the parasites on their own part try to either evade or inactivate the fly's immune responses.

For this reason, what goes on in the midgut between the fly and invading trypanosomes is of strategic importance in the development of trypanosomes in tsetse fly and also in the quest for an effective and reliable control strategy in the fight against African trypanosomiasis. A promising tool in the fight against trypanosomiasis is the use of paratransgenetic approach to modulate the capacity of the fly to acquire trypanosomes (Cheng and Aksoy, 1999), thereby preventing the transmission of the parasites to mammalian hosts. The midgut of the fly therefore represents an important point at which to disrupt the life cycle of trypanosomes, and hence the transmission of the parasites by the tsetse fly vector.

The advent of next-generation sequencing, in addition to its low cost, has enhanced our understanding of the complexity of gene expression, regulation and biological pathways as a result of increased transcript coverage (Tariq et al., 2011). The 454 sequencing platform is fast and offers increased sequencing depth of coverage (Metzker, 2009). The analysis of differential expression of genes between different experimental conditions, such as differential gene expression analysis between uninfected tsetse flies and flies that cleared their parasites following challenge with trypanosomes can be accomplished with 454

sequencing platform. A 454 sequencing platform was used to sequence midgut cDNA from naive flies and flies that cleared their infection following *T. b. brucei* challenge. In this chapter, a bioinformatics analysis of four immune related candidate genes, GMOY000153 (Chitinase, *CHIT*), GMOY002400 (O-GlcNAc transferase, *OGT*), GMOY009713 (secreted phospholipase A_2, *sPLA*$_2$) and GMOY006016 (serine proteinase inhibitor, *SPI*) that were noted to be differentially expressed in the midgut of flies that cleared the infection after being challenged with *T. b. brucei* is presented.

3.2 Materials and methods

3.2.1 Differential gene expression between uninfected and self-cured flies

A transcriptome data from a tsetse midgut 454 library consisting of uninfected flies that were fed normal blood and flies that cleared the parasites following challenge with *T. b. brucei*, strain TSW196 was kindly provided by Dr. Alistair Darby (UoL) for analysis. A tagwise dispersion analysis of the two midgut libraries using the bioconductor package, edgeR (Robinson et al., 2010) was performed by Alistair Darby which revealed a total of 184 differentially expressed genes ($p < 0.05$) out of which 54 top-ranking ones were selected based on highest fold change using arbitrary cut-off values of 1.15 for down-regulated and 1.2 for up-regulated. Accession numbers of the differentially expressed genes were used to retrieve their protein sequences from NCBI and Gene Ontology analysis was carried out using Blast2GO (Conesa et al., 2005) to reveal the functional categories of these genes.

3.2.2 Sequence analyses

Nucleic acid and protein sequences for GMOY000153 (Chitinase), GMOY002400 (O-GlcNAc transferase), GMOY009713 (phospholipase A_2) and GMOY006016 (serine proteinase inhibitor) were downloaded for analysis from the *G. m. morsitans* genome assembly database in VectorBase (https://www.vectorbase.org/) and Geneious Basic (Kearse et al., 2012). The sequences were analysed for the presence of post-translational modifications and conserved motifs. The presence of potential signal peptides were analysed using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) and TargetP 1.1 server (http://www.cbs.dtu.dk/services/TargetP/). The membrane topologies were predicted using the TMHMM server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and Phobius (Kall et al., 2004), a combined transmembrane topology and signal peptide predictor. The protein sequences were analysed to identify functional domains using various programmes including the Simple Modular Architecture Research Tool (SMART) (Schultz et al., 1998, Letunic et al., 2014), the PROSITE dictionary of protein

sites and motif patterns (Gasteiger et al., 2003), PATTINPROT (Combet et al., 2000), a programme used to identify recurring patterns in proteins and TPRpred, a tool for prediction of sequence repeats from protein sequences (Biegert et al., 2006). Also the NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos/) was used to predict the presence of phosphorylation sites.

3.2.3 Phylogenetic analyses

In order to investigate the evolutionary relationship of the candidate genes to various species, the predicted protein sequences of *G. m. morsitans* chitinase (GMOY000153), OGT (GMOY002400), PLA_2 (GMOY009713) and SPI (GMOY006016) were aligned using MUSCLE with corresponding genes from other species obtained from GenBank NCBI, a bootstrapped maximum likelihood tree was generated using PhyML 3.0. The analysis was carried out using Phylogeny.fr (Dereeper et al., 2008), a web-based service dedicated to reconstruction and analysis of phylogenetic relationships between molecular sequences.

3.2.4 Homology modelling

Homology modelling was carried out to predict the three dimensional (3D) structures of GMOY000153, GMOY002400, GMOY009713 and GMOY006016. The amino acid sequences of the genes were downloaded from VectorBase and uploaded separately to Phyre2 (Kelley and Sternberg, 2009), ModWeb (Eswar et al., 2003), CPHmodels 3.2 (Nielsen et al., 2010) and RaptorX (Kallberg et al., 2012) homology modelling servers. Structure refinement of the predicted models was done by ModRefiner (Xu and Zhang, 2011), while the quality of the predicted protein structures were evaluated using QMEAN (Qualitative Model Energy Analysis). Rampage was also used to depict the Ramachandran plots for each predicted 3D structure after refinement.

3.2.5 Ligand binding site predictions

Cofactor, a comparative algorithm for structure-based biological function annotation of protein molecules (Roy et al., 2012) was used to predict amino acids involved in ligand binding sites.

3.3 Results

3.3.1 Differential gene expression between uninfected and self cured flies

A tagwise dispersion analysis (by Dr. Alistair Darby) of the two midgut libraries (flies challenged with trypanosomes and flies fed normal blood) revealed a total of 184 differentially expressed genes ($p < 0.05$) out of which 55 top-ranking ones were selected based on highest fold change, using cut-off values of 1.15 for down-regulated genes and 1.2 for up-regulated genes (by Alistair Darby). Of these, 38 genes (69%) were down-regulated while 17 genes (31%) were up-regulated (Tables 3.1 & 3.2). Gene Ontology analysis was carried out using Blast2GO (Conesa et al., 2005) to reveal the functional categories of these genes. The genes were assigned to three main categories as follows: 56% were assigned to biological process, 25% to molecular function, and 19% to cellular component for up-regulated genes in flies challenged with trypanosomes (Figure 3.1).

In the same set of flies challenged with trypanosomes, down-regulated genes included 52% of genes involved in biological process, 26% involved in molecular process and 22% cellular component (Figure 3.2). Within each of these categories, genes involved in cellular processes (cell cycle and transmembrane transport proteins), metabolic processes (oxidation-reduction, biosynthetic and lipid metabolism), response to stimuli, immune system processes, single-organism process, biological regulation, signalling, cell death, organelle, and binding were the most abundant (Figures 3.1 and 3.2).

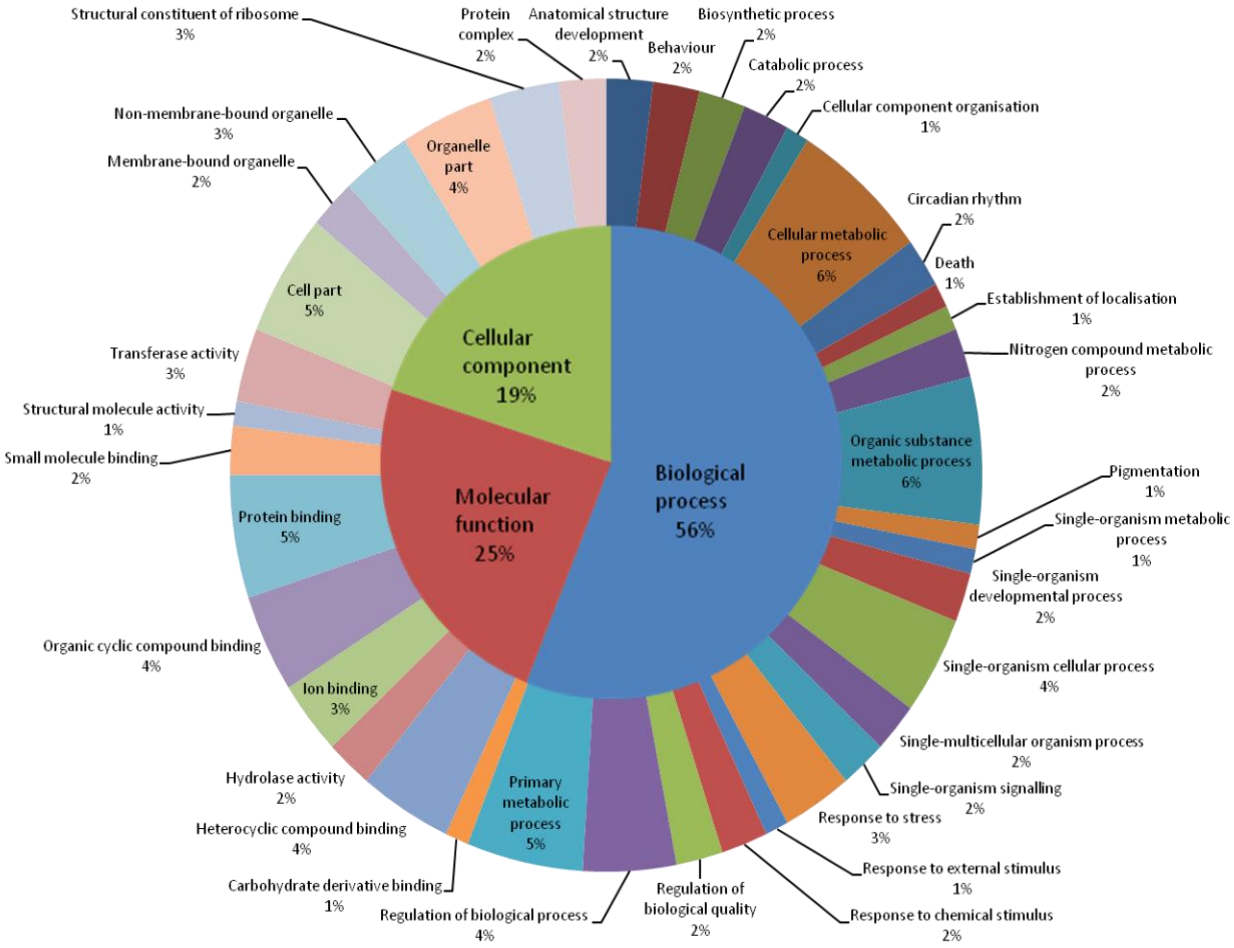

Figure 3.11 Gene ontology (GO) terms distribution of BLAST hits of upregulated genes from refractory flies. Gene ontology analysis was used to categorize the functional distribution of the genes sequenced in this study. For up-regulated genes, 56% were involved biological processes, 25% in molecular function and 19% in cellular component**.**

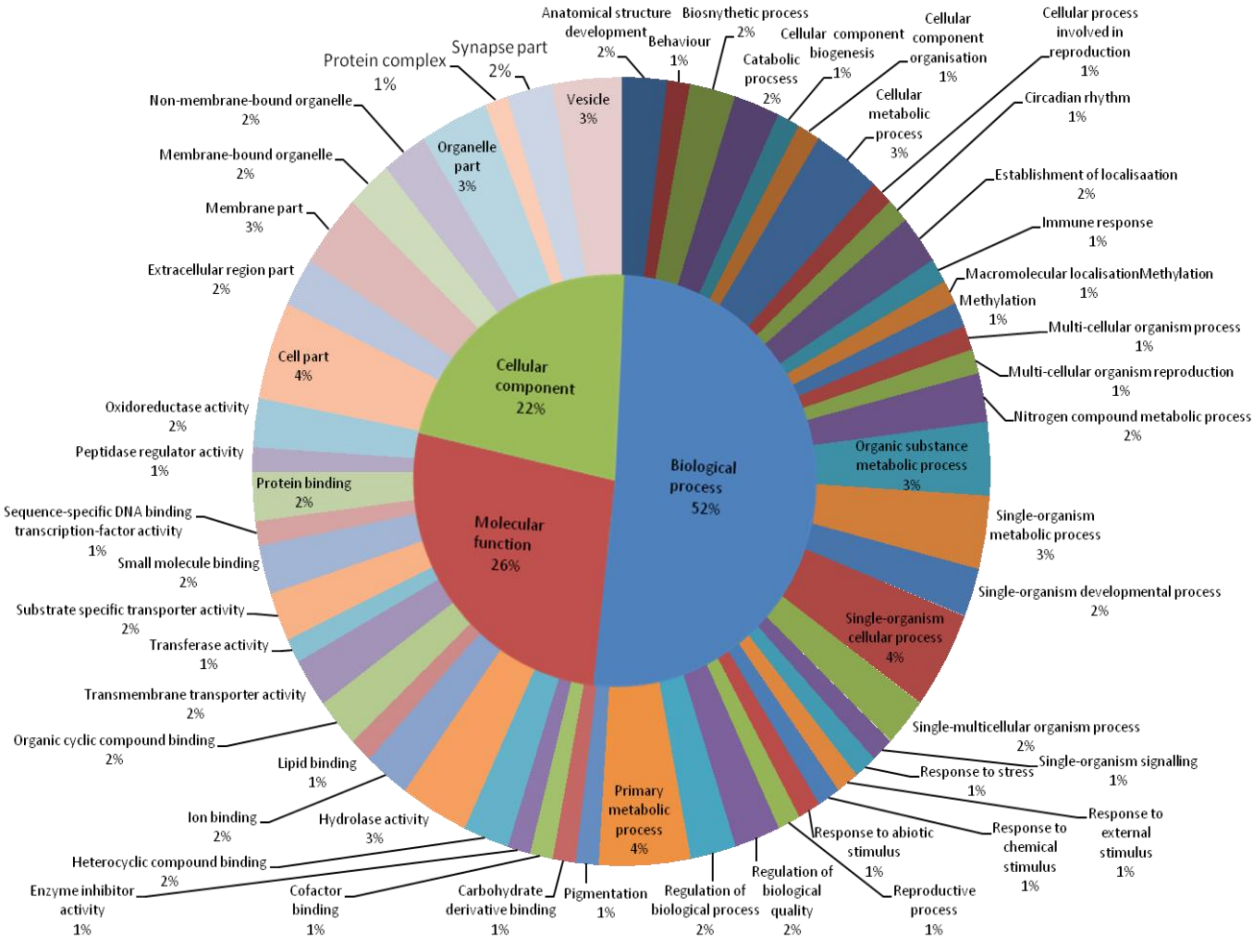

Figure 3.2 Gene ontology (GO) terms distribution of BLAST hits of downregulated genes from refractory flies. Gene ontology analysis was used to categorize the functional distribution of the genes sequenced in this study. For down-regulated genes, 52% were assigned to biological processes, 26% to molecular function and 22% to cellular component.

Table 3.1 List of genes that are downregulated (P<0.05) in trypanosome fed flies. Genes are listed with accession numbers, description and in descending order of fold change (highest fold change at the top)

Gene symbol	Description	Comments
GMOY006073	Homogentisate 1,2-dioxygenase	Oxidoreductase activity
GMOY005501	Synaptic vesicle transporter sVOP	Transmembrane transport
GMOY004234	Fuseless	Clustering of voltage-gated calcium channels, neuromuscular synaptic transmission,neuron-neuronsynaptic transmission
GMOY000550	Hypothetical protein	Sulfate transmembrane transporter activity, secondary active sulfate transmembrane transporter activity
GMOY005361	Hypothetical conserved protein	Hypothetical conserved protein
GMOY012123	Secreted protein	Unknown
GMOY002485	Hypothetical protein	DNA binding
GMOY010709	Kinase suppressor of ras	Protein kinase activity, ATP binding, transferase activity, transferring phosphorus-containing groups
GMOY004228	Transferrin 1	Ferric ion binding, immune related
GMOY009713	Secretory phospholipase isoform a	Phospholipase A2 activity, antibacterial
GMOY006016	Serine proteinase inhibitor	Immune related, Enzyme regulator activity
GMOY000153	Chitinase Chit1 precursor	Immune related, hydrolase activity, acting on glycosyl bonds,
GMOY012079	Aquaporin; integral protein b	Transporter activity
GMOY009897	Pyrimidine metabolism	Nuclease activity, RNA binding, nucleotidyltransferase activity
GMOY007187	Hypothetical conserved protein	Unknown
GMOY004228	Transferrin	Ion binding
GMOY008344	Immune reactive putative protease inhibitor PrInh6	Peptidase activity
GMOY006243	Elongase 1	Long chain fatty acid elongation
GMOY003209	Aldehyde dehydrogenase (NAD+)	Oxidoreductase activity
GMOY002314	Proteoglycan 4-like	Unknown
GMOY010150	Carbon-nitrogen hydrolase	Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds
GMOY007181	Putative uncharacterized protein	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen; haeme binding
GMOY012352	Karmoisin	Transmembrane transport
GMOY010589	U6 snRNA-associatiated Sm-like protein LSm4/Small nuclear ribonucleoprotein Sm D1/D3	RNA biogenesis and function
GMOY003693	M13 family peptidase	Metalloendopeptidase activity
GMOY001312	Glutamic acid-rich protein; Glycoprotein	Chitin binding
GMOY005573	Acid sphingomyelinase	Sphingomyelin phosphodiesterase activity; hydrolase activity
GMOY005361	Hypothetical conserved protein	Unknown
GMOY010934	Glycosyl transferase family 8 glycogenin	Transferase activity, transferring glycosyl groups
GMOY010150	Biotinidase	Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides
GMOY006509	Mitochondrial ribosomal protein pVAR1	Alpha glucosidase
GMOY001312	Glutamic acid-rich protein	Chitin binding
GMOY000943	Hypothetical protein	Protein binding
GMOY009540	Gmfb8d	Unknown
GMOY005442	Retinoid- and fatty acid-binding glycoprotein	Lipid transporter activity
GMOY010266	Hypothetical conserved protein	Haemolymph coagulation-Toll regulated protein in *Drosophila*
GMOY003656	Serpin 4	Peptidase activity

Table 3.2 List of genes that are upregulated (P<0.05) in trypanosome fed flies. Genes are listed with accession numbers, description and in descending order of fold change (highest fold change at the top).

Gene symbol	Description	Comments
GMOY002502	Hypothetical protein	Protein binding
GMOY006920	Hypothetical protein	Nucleic acid binding, zinc ion binding
GMOY002400	Super sex combs	Portein binding
GMOY002761	Hypothetical secreted protein	Unknown
GMOY012049	Heat shock protein cognate 4	Nucleotide binding, ATP binding
GMOY002246	Sphingomyelin phosphodiesterase	Sphingomyelin phosphodiesterase activity, hydrolase activity
GMOY002244	F-box protein	Protein binding
GMOY001552	S-malonyltransferase	Catalytic activity, transferase activity
GMOY008700	Hypothetical protein	RNA binding, protein binding
GMOY004897	Hypothetical protein	Nucloetide binding, nucleic acid binding
GMOY010433	Smell impaired 21F	Unknown
GMOY002485	GK17163	Unknown
GMOY003718	Conserved Zn-finger protein	Unknown
GMOY001280	Starvin	Chaperone binding
GMOY008106	Farnesoic acid O-methyltransferase; Hypothetical conserved protein	Methyltransferase activity, transferase activity
GMOY008065	Decondensation factor 31	Histone binding, chromatin organisation in *Drosophila*
GMOY006187	Hypothetical protein	Unknown

3.3.2 The timecourse of trypanosome elimination in tsetse

Following the ingestion of trypanosomes by tsetse during an infected blood meal, there is the struggle to eliminate the parasites on the part of fly and on the other hand the parasites try to establish an infection by manipulating the fly immune system. From the pattern of expression of most of the immune related genes early on during the infection process, it is hypothesized that following trypanosome ingestion, the immune response of the fly is suppressed by the actions of the parasites to create a favourable environment for establishment of infection. Trypanosomes invade tsetse armed with mechanisms capable of inactivating the fly's immune response. For instance, *T. brucei* are known to possess ISP1 and ISP2 type of serine peptidase inhibitors but lack genes

encoding S1A to which they are highly selective, suggesting that the only targets of these ISPs can only be serine proteases from the tsetse host (Lima and Mottram, 2010). ISP1 and ISP2 are ecotin-like genes found in trypanosomtids and they function as dimeric inhibitors which are highly selective for trypsin-fold serine peptidases belonging to the chymotrypsin family (S1A) (Lima and Mottram, 2010). Host serine proteases such as chymotrypsin-like enzymes found in the gut of the insect vectors are the likely targets of these ISPs (Ramalho-Ortigao et al., 2003, Yan et al., 2001).By inactivating proteolytic enzymes of the host, the parasites are protected from the proteolytic action of the enzymes. Also, *T. b. brucei* are known to be capable of inhibiting tsetse midgut trypsin (Imbuga et al., 1992a). Although tsetse immune defence system already exist before trypanosome invasion, it is expressed at the basic (low) level (Beschin et al., 2014) and therefore easier for the parasites to initiate their development in the fly without much hindrance, at least during the early stages of the infection process before the systematic inducement of the fly defence system. The trypanosomes therefore seem to experience an environment within the tsetse midgut suited to their unrestricted growth during the early days in the infection process after which the tsetse immune system recovers to attack the parasites. This means that priming the fly's immune system prior to trypanosome infection will enhance the elimination of the parasites and this is supported by the evidence that when flies are immune-challenged before trypanosome infection, they become more resistant to trypanosome infection (Hao et al., 2001).

3.3.3 Sequence analyses

GMOY000153 (*CHT*): The 1,570 bp cDNA of GMOY000153 contained 52 nucleotides upstream of the initiation codon, AUG representing the partial 5′ untranslated region (UTR). The open reading frame (ORF) terminates with UAA, i.e., 1382 bases downstream from the initiation codon. The insert, therefore, codes for a polypeptide of 457 amino acids. There are 135 nucleotides after the stop codon, which represents the complete 3′ UTR. Present within the 3′ UTR is the putative polyadenylation signal (PAS) (AAUAAA) (Figure 3.3), followed by

the poly (A) tail, which occurs 24 nucleotides after the PAS signal. The putative protein has a signal peptide with the cleavage site situated between residues 24 and 25 as determined by SignalP v4.1. Also the online tool TargetP v1.1 predicted a signal peptide for the sequence as well as predicting that the protein is secreted. Using the online servers, TMHMM v2.0 and Phobius, residues 1 to 6 stay inside the cell, residues 7 to 25 make up the transmembrane helix, while residues 26 to 240 are outside the cell, thus confirming the presence of a signal peptide in the N-terminal region of the protein. NetPhos 2.0 server predicted the presence of multiple phosphorylated sites (Figure 3.4).

The GMOY000153 protein has the characteristic triosephosphate isomerise (TIM) barrel catalytic domain found in family 18 chitinases which consists of the consensus motif for the active site made up of the amino acid residues FDGIDIDWE (residues, 145 to 153) with the motif DxDxE conserved among all the family 18 chitinase members. It also contains the conserved residues YxR (amino acid residue 182 to 184) of the chitinase insertion domain (CID) (Figure 3.3).

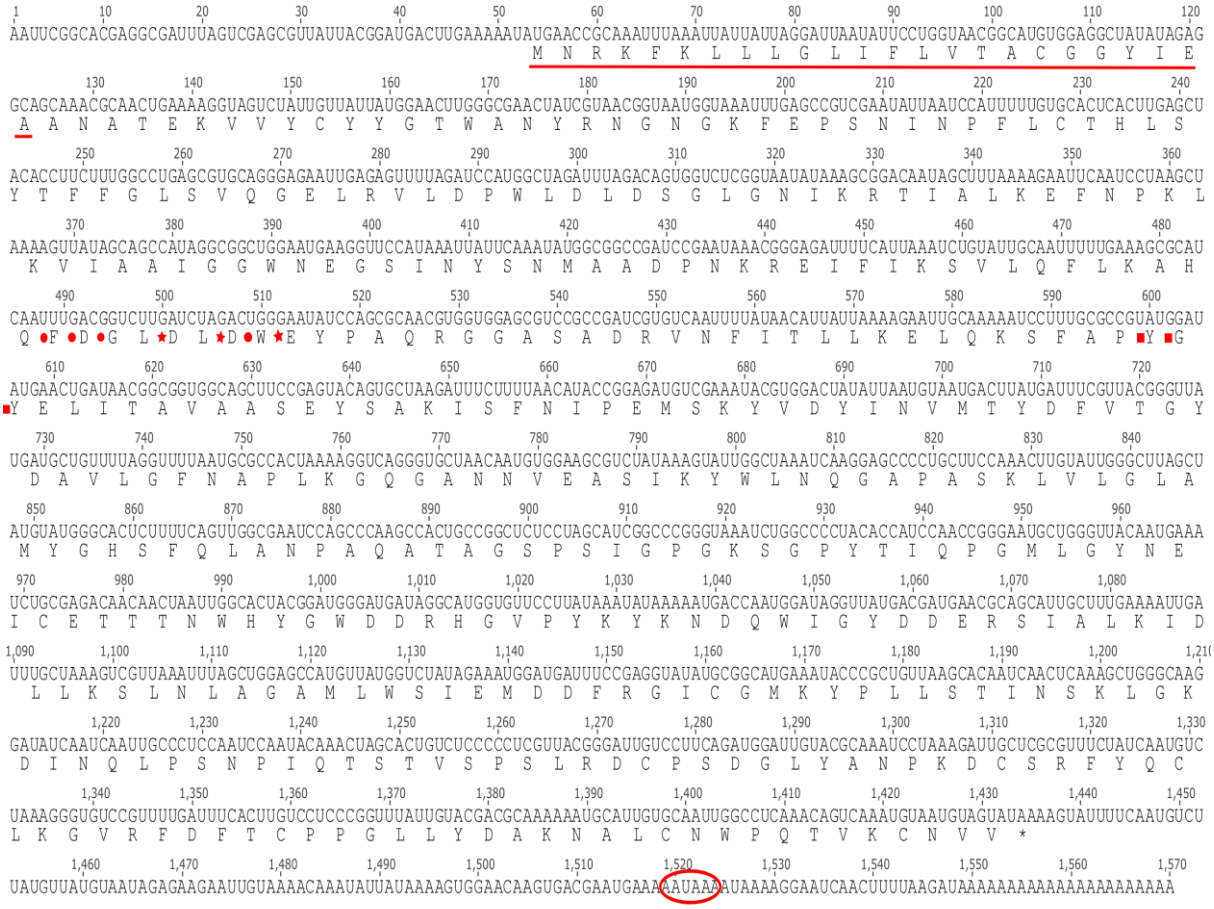

Figure 3.3 Nucleotide sequence and predicted structure of *GmmCHT* . The nucleotide sequence and deduced amino acid sequence of the protein are shown. The underlined regions from residues 1 to 24 represent the signal peptide. The red dots and red stars represent the active site residues while the red squares indicate the conserved protein residues of the chitinase insertion domain (CID). The UAA stop codon is marked in asterisk and the putative polyadenylation signal sequence (AAUAAA) is circled in red.

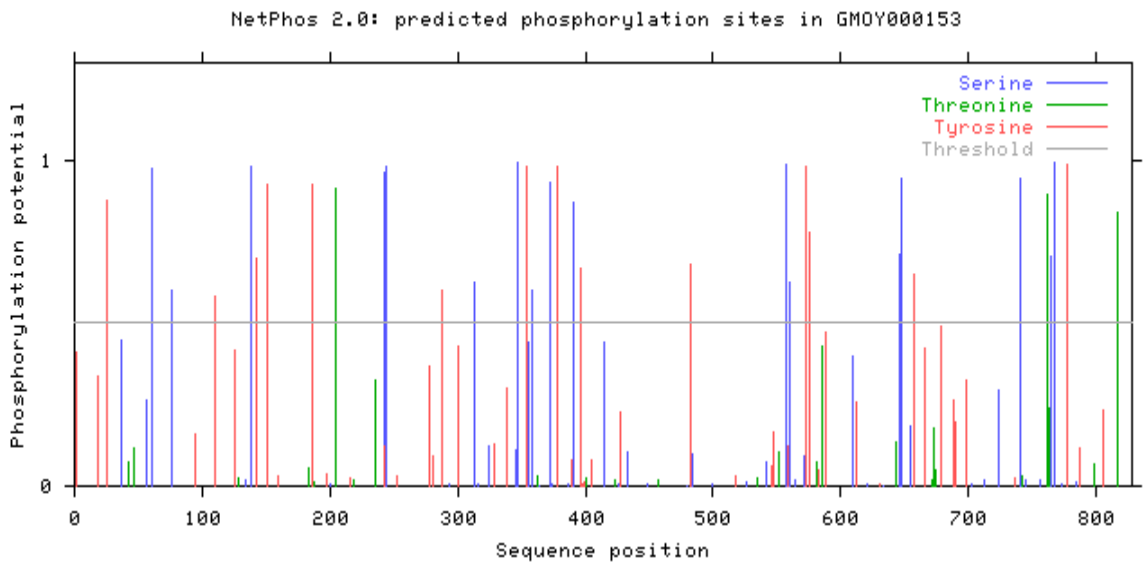

Figure 3.4 Prediction of putative post-translational modifications in *GmmCHT*. The FASTA-formated protein sequence of GMOY000153 from *G. m. morsitans* obtained from VectorBase was entered into the NetPhos 2.0 Server to predict serine (S), threonine (T), and tyrosine (Y) residues that may be phosphorylated. Scores above 0.5 are considered significant. The horizontal line indicates the 0.5 score threshold. Multiple sites were identified as being above the threshold probability value defined by the software.

GMOY002400 (OGT): The *G. m. morsitans* GMOY002400 consists of 4053 base pairs encoding a protein of 1093 amino acid residues. The putative protein has no signal peptide as determined by SignalP 4.1 server and TargetP v1.1. According to NetPhos, GMOY002400 is predicted to be phosphorylated with several residues having prediction scores above the threshold (Figure 3.6). *In silico* sequence analysis identified a tetratricopeptide repeat (TPR)-containing domain between amino acid residues 149-522 (Figure 3.5a). TPR is a versatile structural motif present in a wide range of proteins (Lamb et al., 1995). The TPR motif is made up of 3-16 tandem-repeats of 34 amino acid residues and mediates protein-protein interactions and the formation of protein complexes (D'Andrea and Regan, 2003). PATTINPROT (Combet et al., 2000), a programme used to identify recurring patterns in proteins, TPRpred, a tool designed to detect TPR motifs and other patterns of protein repeats by using the

profile representation of the known repeats and calculates the statistical significance for their occurrence (Biegert et al., 2006) and the Simple Modular Architecture Research Tool (SMART), a biological database used for the identification and analysis of protein domains within protein sequences (Schultz et al., 1998, Letunic et al., 2014), were used to search for TPR sequences. PATTINPROT scans a protein sequence or a protein database against one or several pattern(s).

A query sequence pattern consisting of a template sequence of TPR formulated using the TPR consensus sequence as reported in literature (Lamb et al., 1995, Blatch and Lassle, 1999), was used to search for repeating patterns in our protein of interest. The repeat length is 34 amino acids and repeating time is 3, using different search stringencies and minimum similarity level of 75%, the template specifications based on PROSITE syntax are as follows: X(3)-[WLY]-X(2)-[LIM]-[GAS]-X(2)-[YLF]-X(8)-[ASE]-X(3)-[FLY]-X(2)-[ASL]-X(4)-[PKE]-X(5)-[WLY]-X(2)-[LIM]-[GAS]-X(2)-[YLF]-X(8)-[ASE]-X(3)-[FLY]-X(2)-[ASL]-X(4)-[PKE]-X(5)-[WLY]-X(2)-[LIM]-[GAS]-X(2)-[YLF]-X(8)-[ASE]-X(3)-[FLY]-X(2)-[ASL]-X(4)-[PKE]-X(5), where X represents arbitrary amino acid and the number of repetition is indicated in parenthesis, residues indicated in square parenthesis are those allowed at the position. Eleven equal length tandem repeats (ELTR) TPRs were identified between residues 149-522 (Figure 3.5a) and the consensus sequence that is typical of TPRs are evident (Figure 3.5b). The validity of each identified TPR repeats was further confirmed by going through the individual annotations and conducting individual BLAST searches of each TPR sequence.

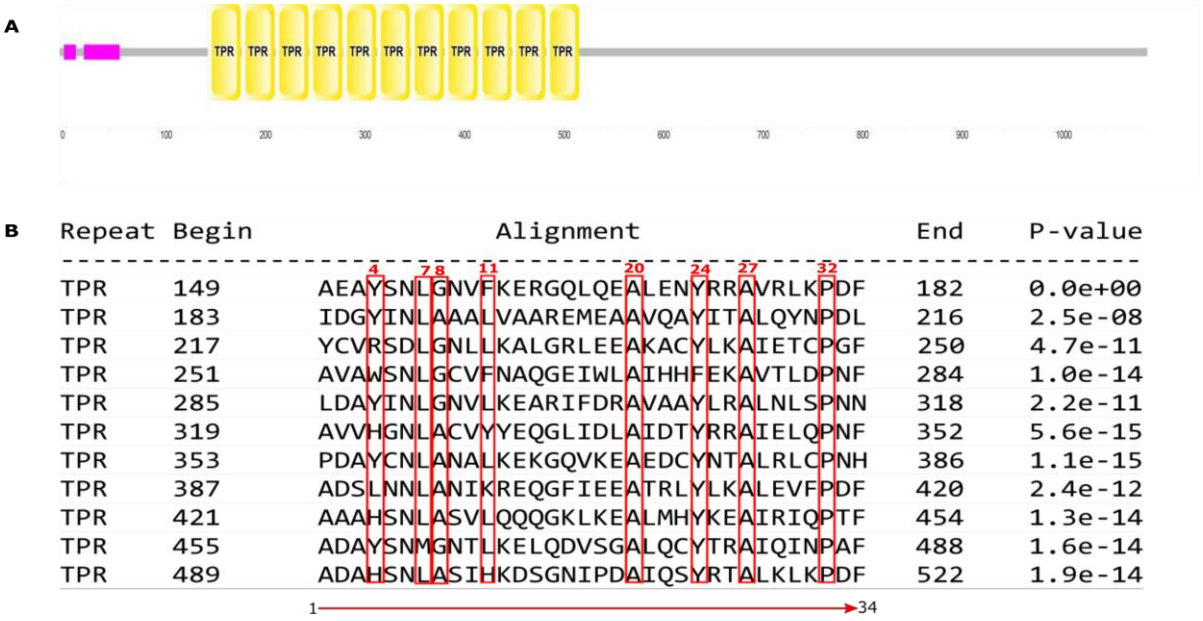

Figure 3.5 The tetratricopeptide repeat region (TPR) of *GmmOGT*. (A) Schematic of the secondary structure arrangement of the 11 TPR motifs of *GmmOGT*. (B) Alignment of 11 TPRs of *GmmOGT*. The positions of the consensus pattern of conserved residues are numbered on top of the alignment. Residue type is highly conserved only at positions 8 (Ala or Gly), 20 (Ala), and 27 (Ala).

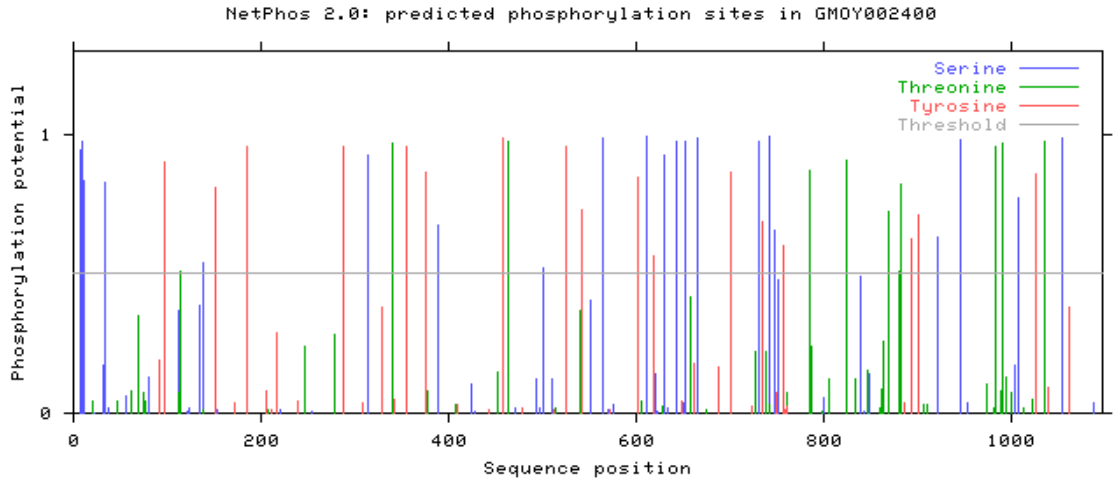

Figure 3.6 Prediction of putative post-translational modifications of *GmmOGT*. The FASTA-formated protein sequence of GMOY002400 from *G. m. morsitans* obtained from VectorBase was entered into the NetPhos 2.0 Server to predict serine (S), threonine (T), and tyrosine (Y) residues that may be phosphorylated. Scores above 0.5 are considered significant. The horizontal line indicates the 0.5 score threshold. Multiple sites were identified as being above the threshold probability value defined by the software.

***GMOY009713* (s*PLA$_2$*):** Bioinformatic analysis of *G. m. morsitans* GMOY009713 revealed 2,600 nucleotide base pairs in which the coding sequence starts at base 152 and ends at base 859, encoding a protein of 236 amino acids (Figure 3.7). Based on analysis using signalP v4.1, the putative protein was predicted to have a signal peptide with the cleavage site between residues 25 and 26. Also the online software TargetP v1.1, predicted that the protein is secreted to the extracellular environment and contains a signal peptide. Using two transmembrane topology predictors, TMHMM Server 2.0 (Krogh et al., 2001) and Phobius (Kall et al., 2004), the putative protein was found to contain no membrane helices. Also it is predicted to be phosphorylated according to NetPhos 2.0 server (Figure 3.6). The *G. m. morsitans* GMOY009713 possesses the consensus sequence found in group XIIA PLA_2 functional domain, which has 3 cysteines, with histidine and asparpartic acid in the catalytic site as conserved residues (**C**-**C**-R-E-**H**-**D**-H-**C**) (Figure 3.6). Another hallmark of the group XIIA PLA_2 (Nevalainen and Cardoso, 2012) found in *G. m. morsitans* GMOY009713 is the Ca^{2+} binding residues in the conserved glycine (residue 109) and the aspartic acid residue in the HD dyad that is found inside the catalytic site. In addition, the protein has 14 cysteine residues that form 7 disulphide bonds which is another conserved feature of group XIIA PLA_2s (Burke and Dennis, 2009).

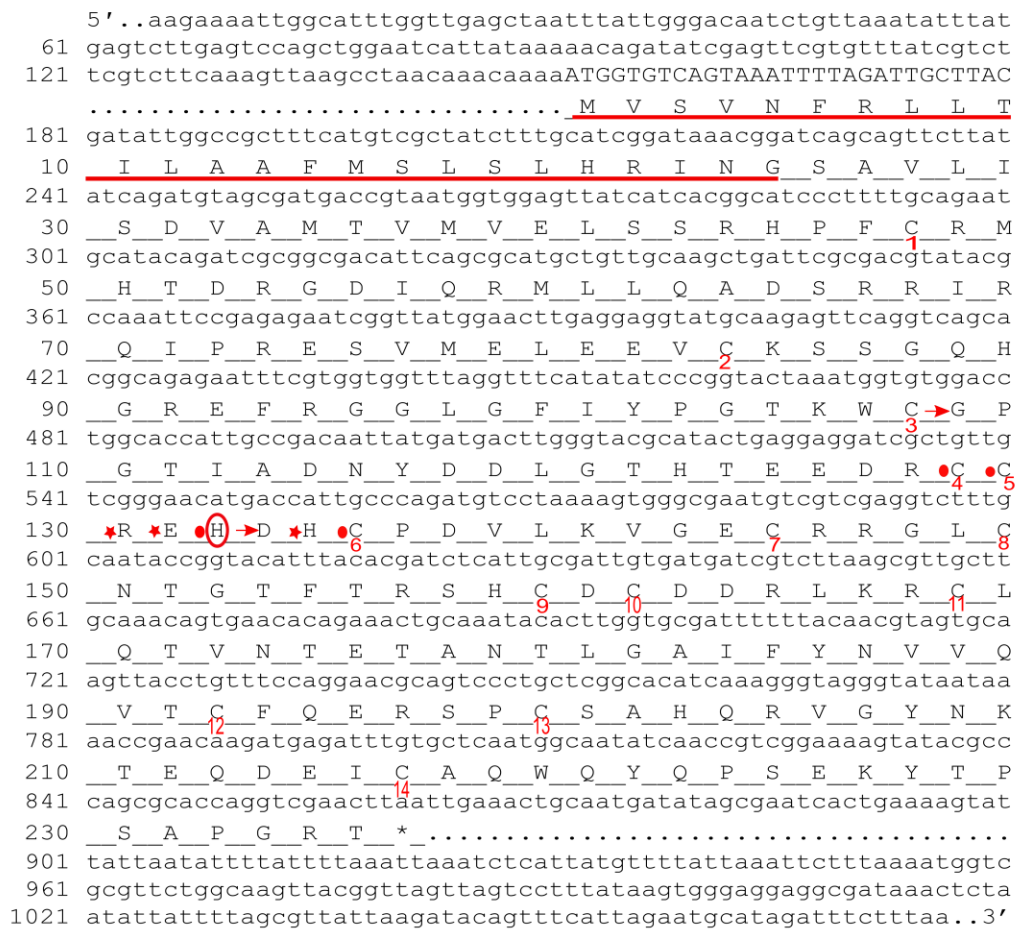

Figure 3.7 Nucleotide sequence and predicted structure of *GmmsPLA$_2$*. Nucleotide sequence of GMOY009713 and deduced amino acid sequence of the protein. The numbering for each sequence is shown on the left. The underlined region (residues 1-25) represents the signal peptide. The red dots and stars represent the PLA_2 consensus sequence with the histidine residue on the active site circled. The 14 cysteine residues that form 7 disulfide bonds are numbered 1-14 while the arrows indicate the Ca^{2+} binding loop residues, both are characteristic features of group XIIA $sPLA_2$s in arthropods.

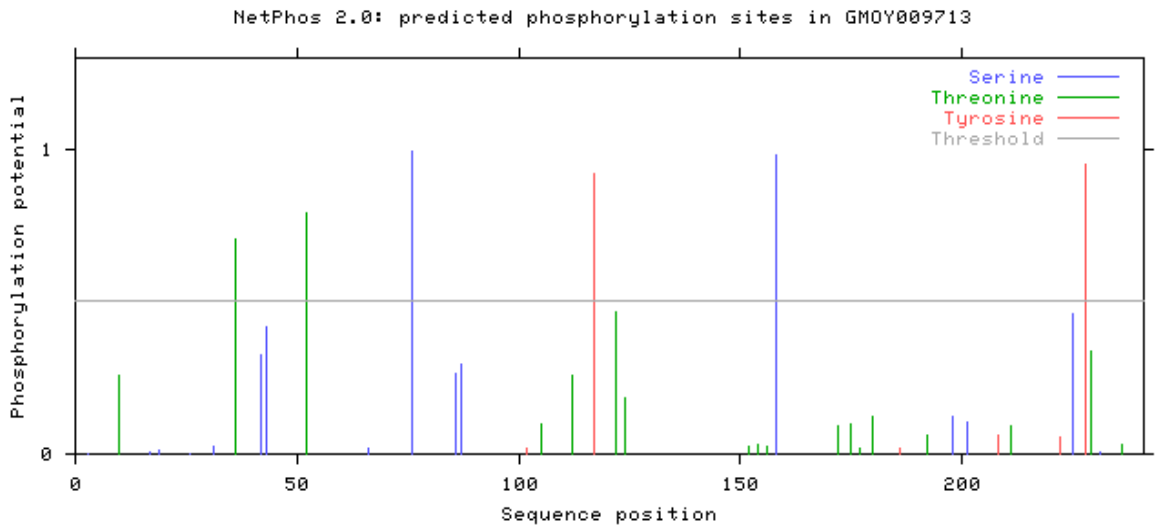

Figure 3.8 Prediction of putative post-translational modifications in GMOY009713. The FASTA-formated protein sequence of GMOY009713 from *G. m. morsitans* obtained from VectorBase was entered into the NetPhos 2.0 Server to predict serine (S), threonine (T), and tyrosine (Y) residues that may be phosphorylated. Scores above 0.5 are considered significant. The horizontal line indicates the 0.5 score threshold. Few sites were identified as being above the threshold probability value defined by the software.

***GMOY006016* (*SPI*):** To characterise the *G. m. morsitans*-derived serine proteinase inhibitor (Serpin), a database search was conducted by running a local BLASTp search against the protein database of *G. m. morsitans* in VectorBase, the corresponding gene which belongs to the kunitz (bovine pancreatic trypsin inhibitor) family contained two Kunitz domains (Figure 3.9). Using SignalP v4.0, a signal peptide was identified with the cleavage site between residues 17 and 18 (Figure 3.9). The online programme TargetP v1.1 also predicted a signal peptide for the sequence and also indicating that the protein is secreted to the extracellular environment. Using TMHMM server v2.0 and Phobius, the protein was found to be devoid of any transmembrane helix. Using NetPhos 2.0 the sequence was found to be phosphorylated (Figure 3.10). Analysis of the peptide sequence showed similarity to other members of the Kunitz-type serine proteinase inhibitor families that are characterised by distinctive features such as the possession of single or multiple kunitz inhibitory domains comprising highly conserved cysteine residues that form disulphide

bridges and a putative P1 (Lys) inhibitory reactive site. According to the protein prediction programme, Scratch Protein Predictor (Cheng et al., 2005), the putative protein is predicted to have two kunitz inhibitory domains with six disulphide bonds formed by twelve cysteine residues at positions 16, 25, 39, 56, 64, 77, 99, 116, 124, 137, 152 and 161 and P1 (Lys 26) residue (Figure 3.9). Predicted disulphide bonds (cysteine pairs) are as follows:

Bond Index	Cys1 Position	Cys2 Position
1	16	25
2	116	137
3	56	77
4	39	64
5	152	161
6	99	124

These features suggest that GMOY006016 is similar to other Kunitz-type serine proteinase inhibitors structurally and functionally.

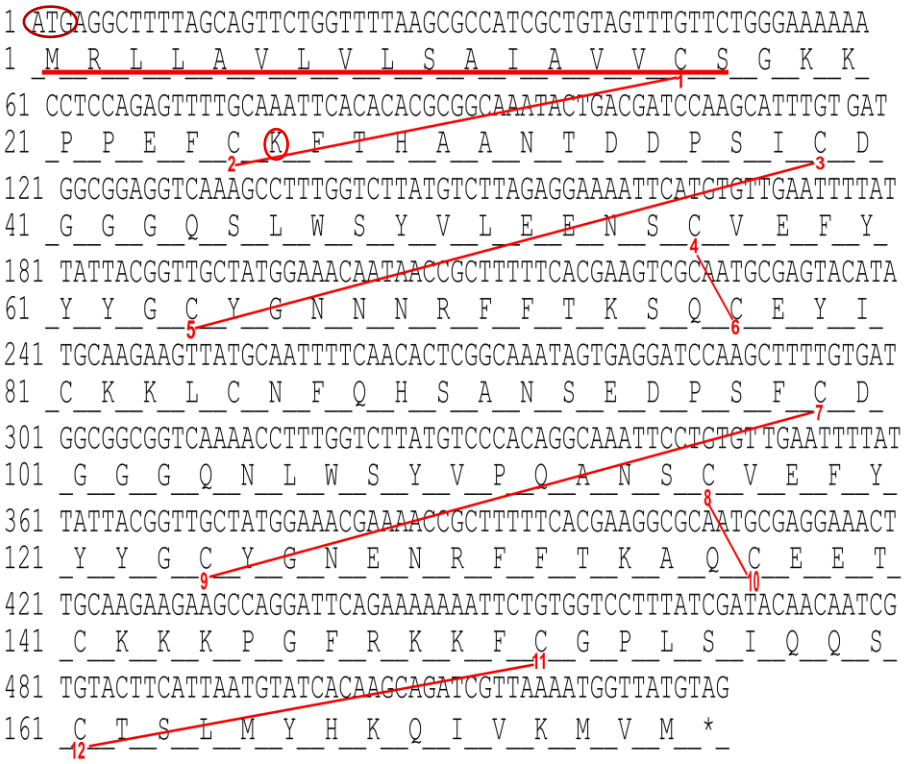

Figure 3.9 The nucleotide sequence and predicted structure of *GmmSPI*. The nucleotide sequence of GMOY006016 and the deduced amino acids sequence are shown. The start codon (ATG) is circled, and the termination codon is indicated with an asterisk. The 12 cysteine residues that form 6 disulfide bonds (representing 2 Kunitz inhibitory domains) are numbered 1-12. The predicted signal peptide (residues 1-16) is underlined while the putative inhibitory reactive site (P1) position is circled.

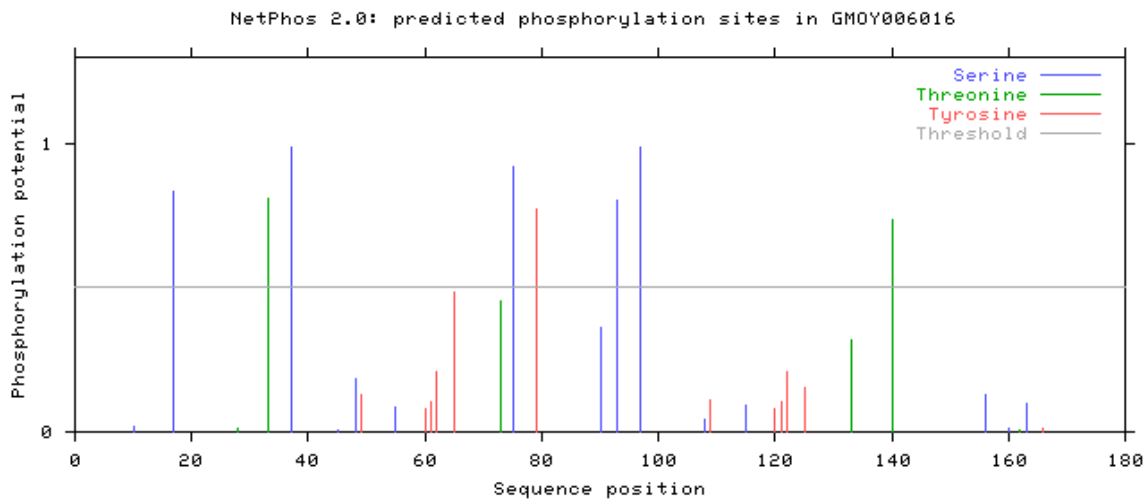

Figure 3.10 Prediction of putative post-translational modifications in GMOY006016. The FASTA-formated protein sequence of GMOY006016 from *G. m. morsitans* obtained from VectorBase was entered into the NetPhos 2.0 Server to predict serine (S), threonine (T), and tyrosine (Y) residues that may be phosphorylated. Scores above 0.5 are considered significant. The horizontal line indicates the 0.5 score threshold. Few sites were identified as being above the threshold probability value defined by the software.

3.3.3 Phylogenetic analysis

A comprehensive analysis was carried out to determine the evolutionary relationship of *G. m. morsitans* chitinase to chitinases derived from various species. The deduced amino acid sequence from GMOY000153 was used to run a BLASTp search against the GenBank NCBI. A total of 34 chitinase sequences (Table 3.3) representing all the major phyla were selected and a multiple sequence alignment was created using MUSCLE. A bootstrapped maximum likelihood tree was created using PhyML 3.0 and further analysis was carried out as described (Dereeper et al., 2008). A summary of the analysis shows that the chitinase sequences were clustered into two main clades, one containing the vertebrates and invertebrates subgroups and the other formed by the bacteria, fungi and viruses subgroups (Figure 3.11). The tsetse chitinase (GMOY000153) is more closely related to *Drosophila* chitinases than to other members of the Diptera, indicating that tsetse and *Drosophila* chitinases had a common origin.

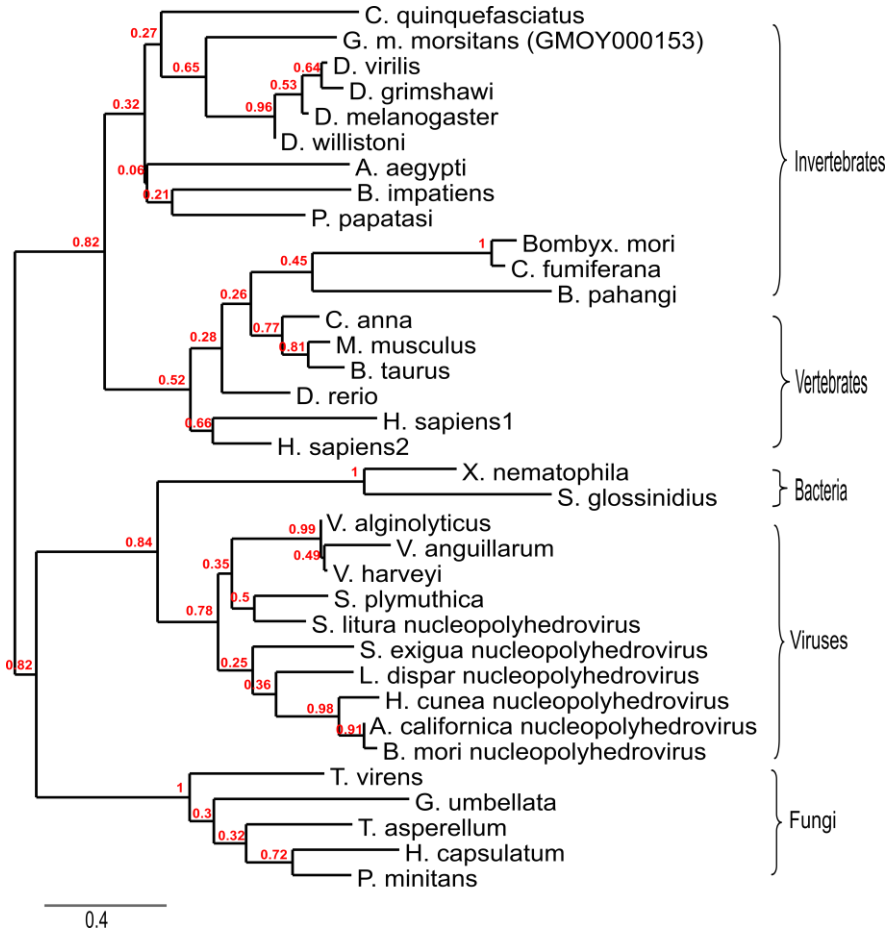

Figure 3.11 Phylogenetic analysis of *GmmCHT* based on amino acid sequence of *G. m. morsitans* and other organisms. Phylogenetic tree was constructed by neighbor-joining criteria with the bootstrap replicates set at 1000. Taxa complete names and the accession numbers of the sequences used in the analysis are shown in Table 3.3.

Table 3.3 List of chitinase and chitinase-like genes used in phylogenetic construction

Gene	Species	Accession Number
Chitinase	*Aedes aegypti*	AAZ39947.1
Chitinase	*Autographa californica nucleopolyhedrovirus*	AAA66756.1
chitotriosidase-1-like	*Bombus impatiens*	XP_003488774.1
Chitinase	*Bombyx mori nucleopolyhedrovirus*	NP_047523.1
microfilarial chitinase, partial	*Brugia pahangi*	AAC47324.1
chitinase precursor	*Bos taurus*	NP_777124.1
chitinase precursor	*Bombyx mori*	BAB20017.1
Acidic mammalian chitinase	*Calypte anna*	KFP05197.1
Chitnase	*Choristoneura fumiferana*	AAM43792.1
chitotriosidase-1	*Culex quinquefasciatus*	XP_001841678.1
GH20466	*Drosophila grimshawi*	XP_001986549.1
Chtinase 4	*Drosophila melanogaster*	NP_524962.2
Zgc:65788 protein, partial	*Danio rerio*	AAH46004.1
GJ21926	*Drosophila virilis*	XP_002050059.1
GK22944	*Drosophila willistoni*	XP_002074826.1
GMOY000153	*G. morsitans morsitans*	AAL65401.1
chitinase, partial	*Grifola umbellata*	AAO42981.1
chitinase	*Histoplasma capsulatum*	AAF80370.1
	Hyphantria cunea nucleopolyhedrovirus	AAD31762.1
Chitinase 3-like 1	*Homo sapiens*	AAH38354.1
chitinase-3-like	*Homo sapiens*	NP_003991.2
chitinase	*Lymantria dispar nucleopolyhedrovirus*	NP_047707.1
acidic mammalian chitinase	*Mus musculus*	AAG60018.1
Chtinase	*Paraphaeosphaeria minitans*	AAG00504.1
midgut chitinase	*Phlebotomus papatasi*	AAV49322.1
Chtinase	*Spodoptera exigua nucleopolyhedrovirus*	AAF33549.1
exochitinase	*Sodalis glossinidius*	CAA72201.1
Chtinase	*Spodoptera litura nucleopolyhedrovirus*	NP_258310.1
Chtinase	*Serratia plymuthica*	CAD32933.1
Endochitinase	*Trichoderma asperellum*	AAF19624.1
Chitinase	*Trichoderma virens*	AAL78812.1
Chitinase B	*Vibrio alginolyticus*	BAB21607.1
Chitinase	*Vibrio anguillarum*	BAA78114.1
chitinase A precursor	*Vibrio harveyi*	AAK11576.1
putative chitinase	*Xenorhabdus nematophila*	CAC38398.1

Phylogenetic analysis was also performed to determine the evolutionary relationship of GMOY002400 with other OGTs from various species. The result of the analysis shows that GMOY002400 is more closely related to *Drosophila* OGTs and shares a more distant common ancestry with OGTs from other arthropods (Figure 3.12). This is expected since there is synteny between *Glossina* and *Drosophila* than other arthropods and they therefore may share a common ancestry (Attardo et al., 2014). Furthermore, a phylogenetic analysis of *G. m. morsitans* sPLA$_2$ (GMOY009713) and representative group XII PLA$_2$s from various species was carried out to determine the evolutionary relationship of GMOY009713 to other group XII PLA$_2$ from various species. The phylogenetic analysis revealed that the members of the group XII PLA$_2$ are highly conserved across the various species and clustered into two main groups namely XIIA and XIIB. As expected, GMOY009713 clustered with other arthropod's group XIIA PLA$_2$s (Figure 3.13), since the arthropods are related to each other and therefore a common ancestor.

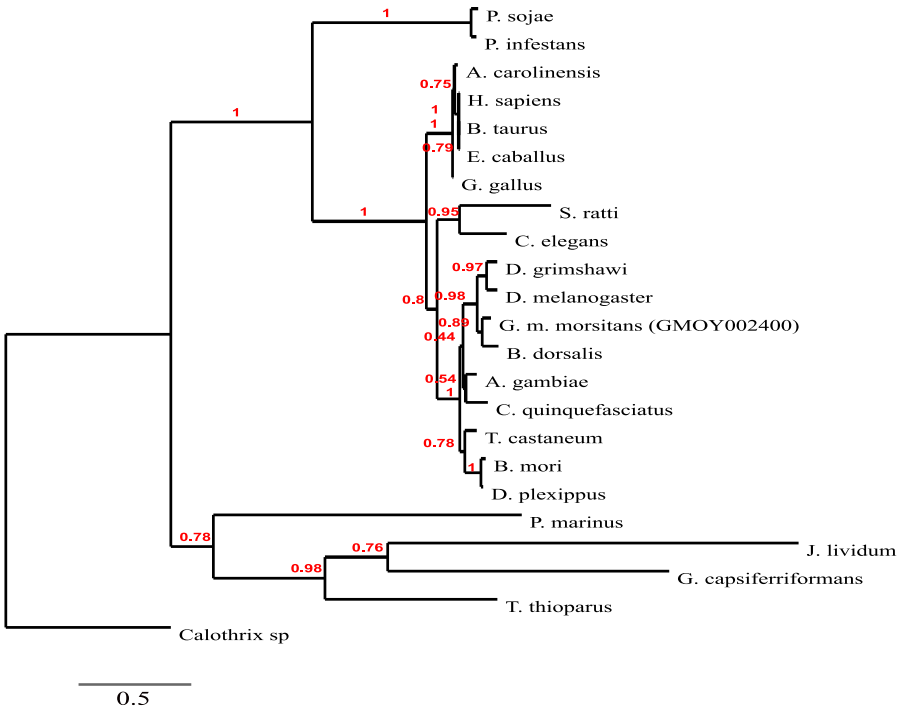

Figure 3.12 Phylogenetic analysis of OGT based on amino acid sequence of *G. m. morsitans* and other organisms. Phylogenetic tree was produced by neighbor-joining criteria with the bootstrap replicates set at 1000. Numbers at the branches correspond to % bootstrap values and the scale represents units

of amino acid substitutions per site. Taxa complete names and the accession numbers of the sequences used in the analysis are shown in Table 3.4.

Table 3.4 List of OGT and OGT-like sequences used in phylogenetic construction

Gene	Species	Accession Number
OGT-1, isoform b	*Caenorhabditis elegans*	NP_001040861.1
OGT	*Bos taurus*	NP_001091539.1
OGT	*Equus caballus*	XP_001493422.1
AGAP006254-PA	*Anopheles gambiae*	XP_316319.4
OGT	*Culex quinquefasciatus*	XP_001846338.1
Super sex combs isoform B	*Drosophila melanogaster*	NP_523620.1
GH21711	*Drosophila grimshawi*	XP_001987067.1
OGT, putative	Phytophthora infestans	XP_002896255.1
OGT, subunit isoform 1	Homo sapiens	NP_858058.1
OGT subunit isoform X1	*Anolis carolinensis*	XP_003228237.1
Hypothetical protein KGM 21815	*Danaus plexippus*	EHJ67792.1
Hypothetical protein	*Prochlorococcus marinus*	WP_011295577.1
OGT subunit-like	*Bombyx mori*	XP_004921733.1
OGT subunit isoform X4	*Gallus gallus*	XP_004940687.1
TPR repeat protein	*Janthinobacterium lividum*	EZP39133.1
OGT subunit isoform X1	*Tribolium castaneum*	XP_008191442.1
Hypothetical protein	*Thiobacillus thioparus*	WP_026240983.1
OGT	*Strongyloides ratti*	CEF64055.1
Hypothetical protein	*Phytophthora sojae*	XP_009514691.1
OGT	*Bactrocera dorsalis*	XP_011205005.1
Hypothetical protein	*Gallionella capsiferriformans*	WP_041938297.1
GMOY002400	*G. m. morsitans*	GMOY002400
Hypothetical protein	*Calothrix sp.*	WP_042341988.1

A phylogenetic analysis of serine proteinase inhibitors made up of SPI protein sequences from arthropods and chordates obtained from GenBank was undertaken in order to elucidate the evolutionary relationships of GMOY006016 with SPIs from various species. Again as expected, GMOY006016 was found to be more closely related to *Drosophila* but shares a more distant common ancestry with the SPI from other arthropods (Figure 3.14).

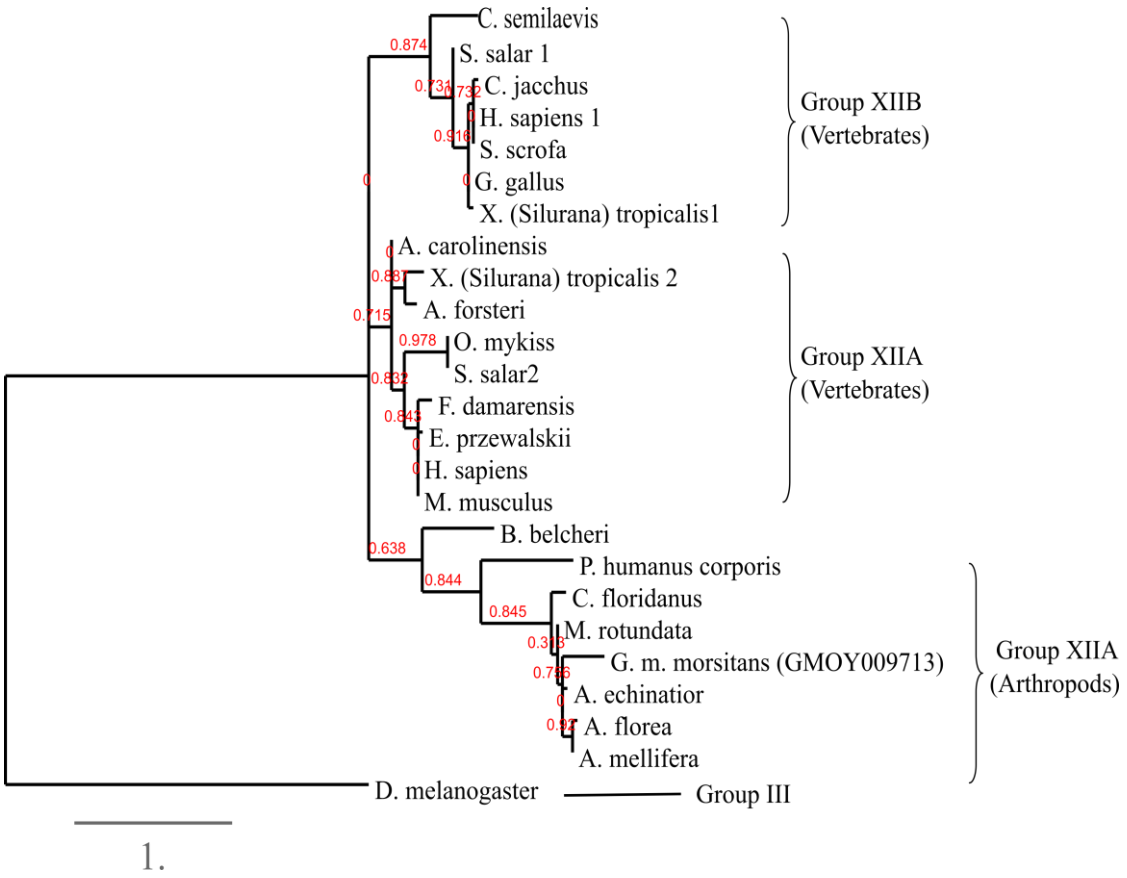

Figure 3.13 Phylogenetic analysis of *GmmsPLA$_2$* based on amino acid sequence of *G. m. morsitans* and other organisms including vertebrates and invertebrates. Phylogenetic tree was produced by neighbor-joining criteria with the bootstrap replicates set at 1000. The tree was constructed with representative sequences selected from vertebrates and arthropods. Taxa complete names and the accession numbers of the sequences used in the analysis are shown in Table 3.5. Numbers at the branches correspond to % bootstrap values and the scale represents units of amino acid substitutions per site.

Table 3.5 List of PLA_2 and PLA_2-like genes used in phylogenetic tree construction

Gene	Species	Accession Number
Predicted groupXIIA PLA2	*Anolis carolinensis*	XP_003221839.2
Group XIIA PLA2	*Acromyrmex echinatior*	EGI70870.1
Predicted GXIIA PLA2-like	*Apis florea*	XP_003694784.1
Predicted GXIIA PLA2	*Arctocephalus forsteri*	XP_009271911.1
Predicted GXIIA PLA2-like	*Apis mellifera*	XP_393116.2
PLA2 group XII	*Branchiostoma belcheri*	AEZ56899.1
Group XIIA PLA2	*Camponotus floridanus*	EFN72747.1
group XIIB PLA2	*Callithrix jacchus*	XP_009007992.1
Predicted GXIIB PLA2	*Carebera semilaevis*	XP_008320501.1
group XIIA PLA2	*Equus przewalskii*	XP_008535645.1
Predicted GXIIA PLA2	*Fukomys damarensis*	XP_010639903.1
Predicted PLA2, GXIIB	*Gallus gallus*	XP_421584.1
PLA2 group XIIA	*Glossina morsitans (GMOY009713)*	ADD19849.1
group XIIA PLA2	*Homo sapiens*	NP_110448.2
group XIIB PLA2	*Homo sapiens*	NP_115951.2
group XIIA PLA_2	*Mus musculus*	EDL12227.1
Predicted GXIIA PLA2-like	*Megachile rotundata*	XP_003699810.1
Group XIIA PLA2	*Oncorhynchus mykiss*	ACO07845.1
GXIIA PLA2	*Pediculus humanus corporis*	XP_002430693.1
Group XIIB PLA2	*Salmo salar*	ACI69856.1
group XIIB	*Sus scrofa*	NP_001230267.1
Group XIIA PLA2	*Salmo salar*	ADM16102.1
PLA2, group XIIA	*Xenopus (Silurana) tropicalis*	AAI57176.1
group XIIB PLA2	*Xenopus (Silurana) tropicalis*	NP_001007917.1
Group III $sPLA_2$	*Drosophila melanogaster*	NP_001285008.1

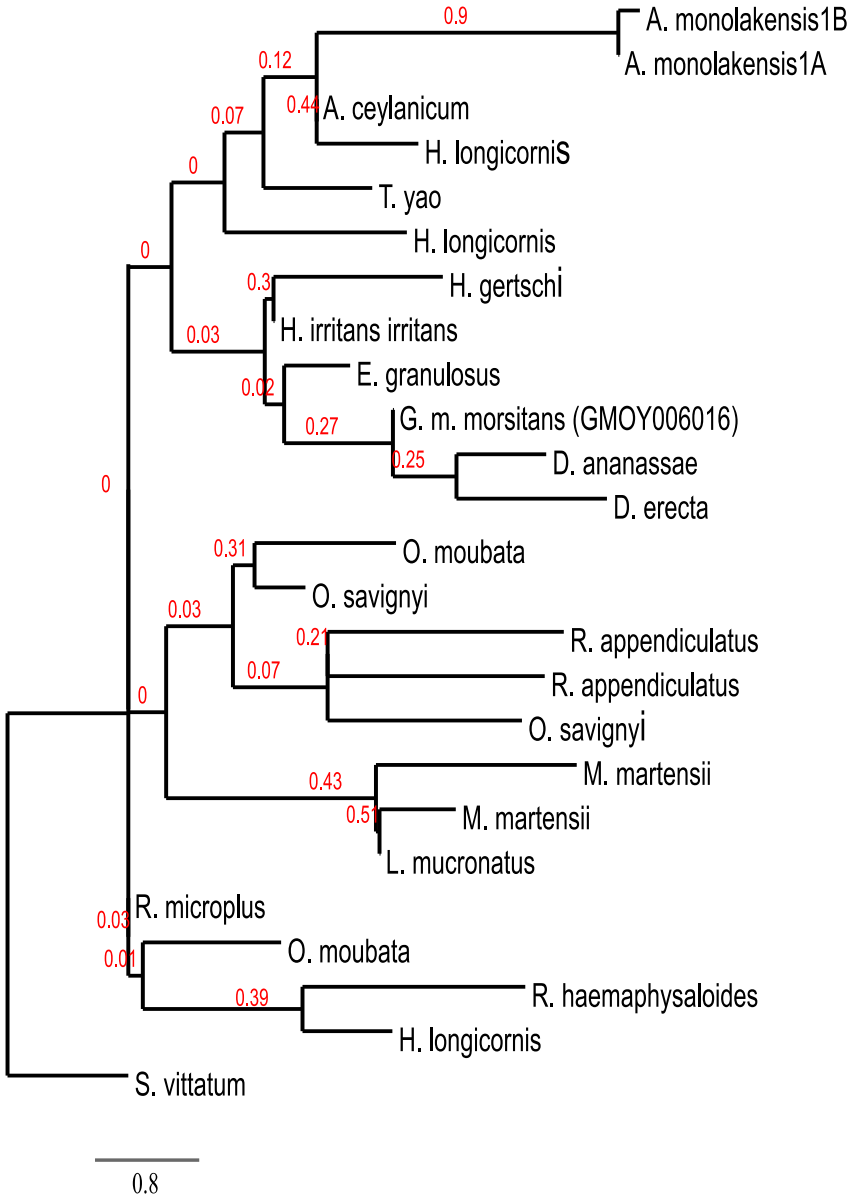

Figure 3.14 Phylogenetic analysis of SPI based on amino acidsequence from G.m. morsitans and other organisms including insects and human. The Phylogenetic tree was constructed with neighbor-joining criteria with 1000 bootstrap replicates. The SPI proteins comprise three major phylogenetic groups. Numbers at the branches represent % bootstrap values. The scale at the bottom left is in units of amino acid substitutions per site. Names and the accession numbers of the sequences used in the analysis are shown in Table 3.6.

Table 3.6 List of SPI and SPI-like genes used in phylogenetic tree construction

Gene	Species	Accession number
monogrin 1A, partial	*Argas monolakensis*	ABI52649.1
monogrin 1B	*Argas monolakensis*	ABI52650.1
Tick anticoagulant peptide (TAP)	*Ornithodoros moubata*	P17726.1
Chymotrypsin inhibitor precursor	*Rhipicephalus microplus*	ACF57858.1
GF16246	*Drosophila ananassae*	XP_001955442.1
GG11203	*Drosophila erecta*	XP_001982147.1
Single kunitz protease inhibitor	*Simulium vittatum*	ACH56928.1
Serine protease inhibitor	*Haemaphysalis longicornis*	BAG82647.1
BPTI-like protease inhibitor, partial	*Haematobia irritans irritans*	AAL87009.1
Savignygrin	*Ornithodoros savignyi*	AAM54048.1
Kunitz protein 8	*Echinococcus granulosus*	ACM79010.1
Kunitz/BPTI-like protein precursor	*Rhipicephalus appendiculatus*	ACM86785.1
Kunitz-type protease inhibitor Hg1	*Hadrurus gertschi*	P0C8W3.1
Serine protease inhibitor 3	*Tabanus yao*	ACS72290.1
fXa inhibitor fXaI	*Ornithodoros savignyi*	AAN76827.1
Kunitz-type trypsin inhibitor	*Haemaphysalis longicornis*	BAI99730.1
Anticoagulant protein rhipilin-1	*Rhipicephalus haemaphysaloides*	ADJ56344.1
Chymotrypsin inhibitor	*Haemaphysalis longicornis*	BAM28739.1
Kunitz-type serine protease inhibitor	*Lychas mucronatus*	P0DJ45.1
Kunitz-type serine protease inhibitor	*Mesobuthus martensii*	P0DJ49.1
Platelet aggregation activation inhibitor	*Ornithodoros moubata*	P36235.1
Tryptase inhibitor precursor	*Rhipicephalus appendiculatus*	AAW32666.1
Kunitz type serine protease inhibitor, partial	*Ancylostoma ceylanicum*	AAD51334.1
Kunitz-type serine protease inhibitor	*Mesobuthus martensii*	P0DJ50.2
Serine proteinase inhibitor	*G. m. morsitans*	GMOY006016

3.3.4 Homology modelling

Four homology modeling servers were recruited to independently predict the 3D structures of GMOY000153, GMOY002400, GMOY009713 and GMOY006016. The most reliable models were those predicted by CHPmodels server (Figure 3.15). The stereochemical qualities of the models predicted by CPHmodels were the highest when compared with all the other models taking into consideration both the Qualitative Model Energy ANalysis score (QMEAN) (Benkert et al., 2008), a composite scoring function describing the major geometrical aspects of protein structures and percentage identity with the template protein (Table 3.7). Refinement of the structures of the predicted models was carried out using ModRefiner to generate initial and refined models. After refinement, the Ramachandran plots of the initial and final (refined) models were generated and compared. All predicted 3D models showed a high percentage of the main chain conformation (between 92.8%-99.0%) of residues to be in the favoured regions, between 1.0%-4.6% were in allowed regions and between 0%-3.4% were in outlier regions as determined by the Ramachandran plot analysis.

Table 3.7 Comparison of homology modelling server performance in the prediction of 3D models. The higher the QMEAN score the better the quality of the protein structure. The percentage identity with template protein and the QMEAN score were highest for the models predicted by CPH.

Gene	Server	Template	% Identity	QMEAN Score
GmmCHT	Phyre2	c3oa5A	30.0	0.528
	Modweb	3fy1A	38.5	0.512
	CPH	3fxy	39.9	0.699
	Raptorx	3fxyA	32.0	0.656
GmmOGT	Phyre2	C3pe3D	73.0	0.574
	Modweb	4gywA	73.0	0.635
	CPH	4n39	73.5	0.671
	Raptorx	3pe3A	72.0	0.567
GmmPLA$_2$	Phyre2	d1pocA	34.0	0.299
	Modweb	1pocA	36.0	0.510
	CPH	1poc	41.0	0.530
	Raptorx	1pocA	40.9	0.498
GmmSPI	Phyre2	C4bd9B	31.0	0.384
	Modweb	3bybA	35.8	0.421
	CPH	4bd9	36.9	0.501
	Raptorx	1bikA	33.8	0.532

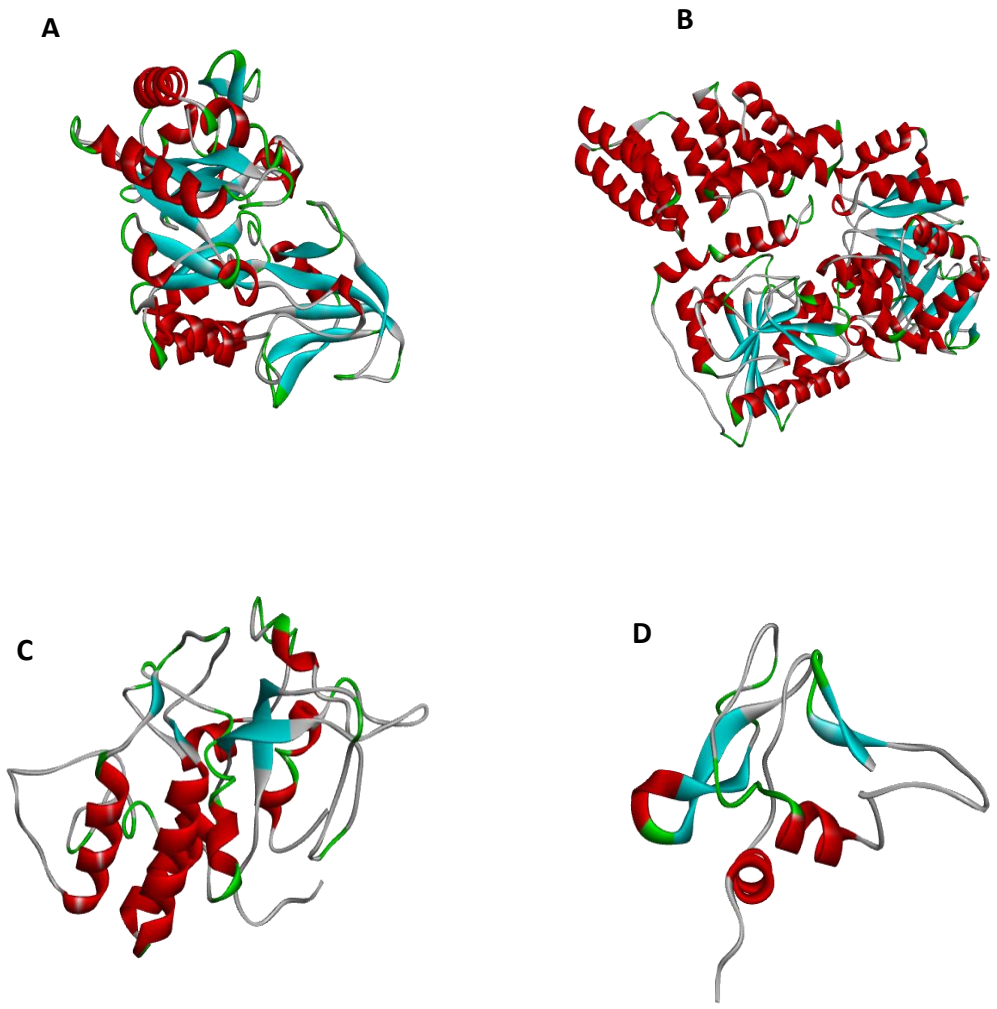

Figure 3.15 Three dimemsional structures of *GmmCHT* (A), *GmmOGT* (B), *GmmPLA$_2$* (C) and *GmmSPI* (D). 3D structures were based on 3fxy, 4n39, 1poc and 4bd9 for A, B, C and D respectively. Helices are in red and β-strands are in blue.

3.3.5 Ligand binding sites predictions

In silico analysis identified putative ligand binding sites in the genes under investigation (Figure 3.16). For GMOY000153 there are 15 amino acid residues in the binding site, for GMOY002400, 8 amino acid residues, for GMOY009713, 11 amino acid residues and for GMOY006016, 9 amino acid residues (Figure 3.16).

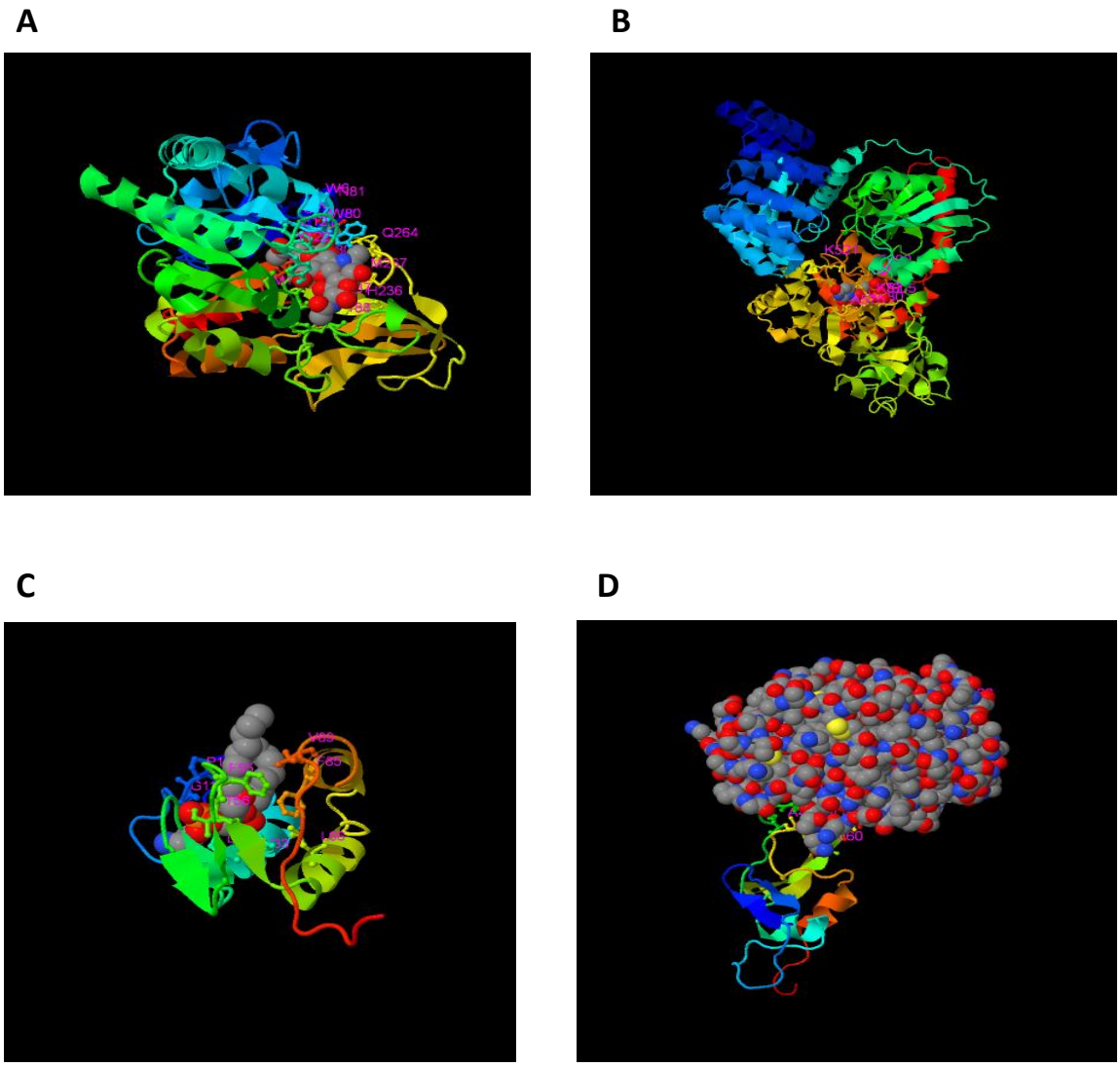

Figure 3.16 Ligand binding sites residues for *GmmCHT*, *GmmOGT*, *GmmsPLA$_2$* and *GmmSPI* as predicted by COFACTOR. Predictions were based on template proteins with similar binding sites to Chitinase, OGT, PLA_2 and SPI. (A) *GmmCHT* contains multiple ligands and 15 putative binding site residues (B) *GmmOGT* contains a uridine-5'-diphosphate (UDP) ligand and 8 putative binding site residues (C) *GmmPLA$_2$* contains GEL ligand and 11 predicted binding site residues (D) *GmmSPI* contains peptide ligand and 9 putative binding site residues. Proteins are shown in ribbons and ligands in space filling models.

3.4 Discussion

Genes that were involved in various pathways including immune response, metabolism, cell death, cellular and cell signalling were found to be differentially expressed in flies that cleared infection after being challenged with trypanosomes (Figures 3.1 and 3.2). Since tsetse flies become more resistant to trypanosome infection if they are fed normal blood before being infected (Welburn and Maudlin, 1999), flies were fed infectious blood containing blood stream forms of *T. b. brucei* at the fourth blood meal to reproduce the refractory phenotype and dissected 3 days after receiving the infective blood meal. It has been shown that the rate of midgut infection in flies infected at this stage is low – less than 5% (Distelmans et al., 1982, Haines et al., 2010). Also, infected flies were dissected at a time (day 3 post-infection) that coincides with the occurrence of major events in the midgut in tsetse-trypanosome interaction leading to complete elimination of infection in a proportion of flies (Gibson and Bailey, 2003) to increase the opportunity of identifying genes that play important roles during the time the fly tries to eliminate the invading trypanosomes.

The transcriptome data comprised of reads from control (uninfected) and flies fed an infective bloodmeal (containing a mixture of self-cured (refractory) and infected (susceptible) flies). Comparison of gene expression was therefore between uninfected flies and flies challenged with trypanosomes. It would have been ideal to also compare gene expression between susceptible and refractory flies, however susceptibility to trypanosomes introduced at the 4th bloodmeal is typically less than 5% (Haines et al., 2010, Distelmans et al., 1982). Consequently, this group is deemed naturally refractory to infection.

Pathogens have evolved various strategies to infect and colonise their hosts by evading the immune defences mounted by the hosts (Munter et al., 2006). Insects mount a robust innate immune response when confronted with pathogens (Christophides et al., 2002). The insect innate immune system is complex and involves a wide range of gene families involved in various pathways. Although the innate immune defence system of tsetse is active prior to trypanosome invasion, it exists at the basic level which may not be sufficient to deal with the parasites which are already armed with mechanisms capable of

inactivating the tsetse immune response. This may tilt the molecular war between tsetse and trypanosomes in favour of the parasites, especially during the early stages of the infection process. The genes expressed in the midgut of the fly during trypanosome infection are of vital importance since the midgut is the first port of call for pathogens and where the struggle for survival commences.

The first and most decisive step in the life cycle of the trypanosome in the tsetse fly is its establishment in the midgut of the fly. It is in the midgut of the fly that the processes of elimination starts after the bloodstream forms ingested when taking an infected blood meal differentiate into procyclics within the first 3 days. During this period, most flies within a population are able to eliminate the parasites while very few become susceptible (Gibson and Bailey, 2003, Aksoy et al., 2003, Van Den Abbeele et al., 1999). This process is by no means straightforward because the parasites on their part try to increase their chance of survival by inactivating or evading the immune response of the fly. This results in a molecular crosstalk between the fly and the invading parasites.

The 454 sequence data reveals genes that were differentially regulated in the midgut of tsetse flies that were challenged with trypanosomes. The common classes of genes revealed include serine proteases and serpins, genes encoding for PM adhesion proteins, genes involved in immune defenses, reactive intermediates and genes whose functions are not known.

A serine proteinase was observed to be up regulated while there was a down regulation of protease inhibitors (two serine proteinase inhibitors, a metalloendopeptidase and one chymotrypsin inhibitor) in flies that were challenged with trypanosomes. The proteases of invading pathogens as well as the endogenous proteases have the potential to cause undesirable destructive action if allowed to act beyond their intended target. As a result protease inhibitors have evolved to play regulatory roles to keep the action of endogenous proteases in check (Armstrong, 2006). Also the inactivation of secreted proteases of invading pathogens could have negative impact on their invasion and proliferation as well as deprivation of nutrients to those parasites that have already invaded the host (Armstrong, 2006) thereby protecting the host from pathogenic infections.

It is not surprising therefore that there was a down-regulation of serine proteinase inhibitor (SPI) in flies challenged with trypanosomes since the flies will try to secrete more protease to destroy the parasites. The parasites on the other hand will try to prevent the inactivation of their protease by suppressing the host serine proteinase inhibitors in order to create a favourable environment in which to thrive. There was a down regulation of both aquaporin and lipophorin in the flies that were infected with trypanosomes. This is in variance to what has been observed in *Ae. aegypti* where aquaporin is over expressed in response to infection with alphaviruses (Sanders et al., 2005) and the up regulation of lipophorin in *Ae. aegypti* when infected with Gram-positive bacteria, fungal spores and *P. gallinaceum* (Cheon et al., 2006). The down regulation of both aquaporin and lipophorin in flies challenged with trypanosomes could be attributed to the fact that majority of the flies must have eliminated the trypanosomes which leads to a decrease in the level of expression of these genes that are known to be induced upon immune challenge. However, it should be noted that gene expression was analysed at just one timepoint (flies were dissected at 3 dpi). It would have been ideal to sample the pattern of gene expression at multiple timepoints during the infection process. Doing this would have presented a more robust argument as to the significance of the difference in gene expression. In *Ae. aegypti*, the survival and proliferation of virus is thought to be aided by the presence of alpha glucosidase in the midgut (Tchankouo-Nguetcheu et al., 2010). There was an up regulation of a putative acid sphingomyelinase (sMase), a gene that is involved in programmed cell death (PCD) and stress signalling (Won and Singh, 2006). sMase is involved in the synthesis of sphingolipids with the production of pro-apoptotic ceramide a key regulator of programmed cell death (Mullen and Obeid, 2012). Acid sphingomyelinase is implicated in the killing of bacteria and the initiation of apoptosis in infected host cells (Becker et al., 2010). It is therefore possible that infected flies up-regulate acid sphingomyelinase to kill the trypanosomes.

O-Linked β-N-Acetylglucosamine transferase (*OGT)* was up-regulated in flies that were challenged with trypanosomes but that later cleared their infection. The enzyme OGT catalyses the addition of a single *O*-GlcNAc linkage to serine or threonine residues of nuclear and cytoplasmic proteins which are involved in a

wide range cellular functions (Hart et al., 2007). Overexpression or knockdown of OGT has been proven to affect a wide range of signalling pathways and cellular processes that include insulin signalling/diabetes, stress response, cell cycle regulation, cell regulation and immune response (McClain et al., 2002, Zachara et al., 2004, Slawson et al., 2005, Golks and Guerini, 2008). OGT is involved in the modulation of cellular processes with regards to nutrient availability, stress, immune regulation and cell cycle regulation (Paula et al., 2012). One important biological stressor is parasitic infection (Beckerman et al., 2013). Also increasing the expression of O-GlcNAc enables cells to become more tolerant to stress and improves cell survival (Chatham et al., 2008, Laczy et al., 2009). The up-regulation of OGT in flies that has been challenged with *T. brucei* could be a mechanism employed by tsetse to fight back infection, and the increase in infection rate following RNAi-mediated knockdown of OGT suggests that OGT plays a role in immune response in infected flies.

There was a down-regulation of chitinase in flies challenged with trypanosomes compared to flies that received normal blood. Chitin and chitinases have been shown to be associated with host immune response in a variety of organisms ranging from plants to mammals (Tiffin and Moeller, 2006, Shi and Paskewitz, 2004, Kramer, 2009, Nair et al., 2003, Lee et al., 2008). In *Anopheles gambiae* chitinase-like proteins are thought to be involved in tissue remodelling and/or immune responses (Shi and Paskewitz, 2004). Key features of the arthropod innate immune system such as antimicrobial peptides, melanisation and phenoloxidase (PO) cascade (Soderhall and Cerenius, 1998, Bulet et al., 1999), are thought to be influenced by chitin metabolism. The production of melanin, a key feature of invertebrate immune system has been linked to chitin metabolism (Walker et al., 2010). Chitinase which is directly involved in chitin metabolism (Muthukrishnan et al., 2012) can be said to play an important role in insect immune response. The down-regulation of chitinase in trypanosome challenged flies could be a ploy by the parasite to suppress the immune response of tsetse in order to establish an infection in the midgut since the thickness of the chitin-rich peritrophic matrix which is regulated by gut-specific chitinases (Filho et al., 2002) could be compromised and allow the parasites to pass through without much hindrance Also it has been suggested that in arthropods the chitin

synthesis and degradation pathway is central to the regulation of defences and therefore allow an organism to defend itself against multiple threats (Beckerman et al., 2013).

3.4.1 What does sequence analysis tell us about the structure and function of DE genes expressed in refractory flies?

Conserved protein residues are often seen in protein families where they play important functional roles as well involvement in critical stabilizing interactions since evolutionary pressure for stability or function could lead to clustering of conserved residues (Schueler-Furman and Baker, 2003). Also residues important for stability are often clustered together in the hydrophobic core and functional residues may be close together in sites involved in enzymatic reactions, protein-protein interaction or ligand binding sites (Schueler-Furman and Baker, 2003). Analysis of residue conservation is therefore a sensible approach to identify functionally important sites in a sequence.

GMOY000153 contains the conserved consensus sequences of family 18 chitinases consisting of the conserved active motif (DxDxE) of the catalytic domain. Structure prediction showed GMOY000153 has a triosephosphate isomerise fold or TIM barrel. This is typical of the catalytic domain of family 18 chitinases (Suzuki et al., 2002). Also the catalytic domain contains a chitinase insertion domain (CID) which incorporates the conserved amino acid residues YxR that is known to interact with the substrate (Li and Greene, 2010). This small domain which is inserted into the TIM barrel catalytic domain is present in chitinases of subfamily A, but not in subfamily B (Suzuki et al., 2002). The CID has a large percentage of aromatic residues most of which exist in the hydrophobic core except those residues which interact with sugar and this may play an important role in the folding and stability of the protein (Li and Greene, 2010). It is known that aromatic residues play an important role in the structural stability of proteins and peptides (Palermo et al., 2008, Subramaniam et al., 2001). Therefore the presence of the CID and the TIM barrel may contribute to the thermal stability of the whole enzyme (Li and Greene, 2010).

Sequence analysis revealed that GMOY002400 contains a tetratricopeptide repeat (TPR)-containing N-terminal domain which consists of a variant consensus sequence typical of TPRs (Figure 3.5B). Although the consensus sequence defines the TPR, and consists of a mixture of small and large hydrophobic amino acid residues, no positions are fully invariant (Zeytuni and Zarivach, 2012). However, certain residues were found to be conserved including positions 4, 7, 8, 11, 20, 24, 27, and 32, but only positions 8 (Alanine or Glycine), 20 (Alanine), and 27 (Alanine) were highly conserved and consensus position 32 which is located in the turn between two TPR motifs is occupied by proline, (Figure 3.5B). This bears the hallmark of a TPR domain which consists of highly conserved residues at positions 8, 20, and 27, whereas positions 4, 7, 11, and 24 show stronger preference for large hydrophobic amino acids with position 32 being usually occupied by a helix-breaking residue such as proline (D'Andrea and Regan, 2003).

TPR is a versatile structural motif present in a wide range of proteins from bacteria to humans and are involved in diverse cellular processes such as transcriptional control, protein folding, transport and degradation, host defence against invading pathogen and viral replication (Lamb et al., 1995, Goebl and Yanagida, 1991, Callahan et al., 1998, Cziepluch et al., 1998, Mamane et al., 2000). It is made up of 3-16 tandem-repeats of 34 amino acid residues which mediates protein-protein interactions with the formation of multi-protein complexes which elicits varying biological roles (D'Andrea and Regan, 2003). As a result of the numerous interactions that TPR-proteins are involved in and the diversity of the TPR domain, they hold a great promise for protein engineering, therapeutics and biotechnology since their binding specificity and affinity towards ligands of interest can be modulated by redesigning the basic TPR scaffold. Ligands with higher affinity can be designed to inhibit or isolate the TPR domains from the proteins to which they are usually attached and this can be employed to disrupt protein-protein interactions within the cell.

Sequence analysis of GMOY009713, a protein of 236 amino acids, revealed the presence of several conserved features of group XII PLA_2s. When phylogenetically compared to other PLA_2s from other species, GMOY009713 shared homology with GXIIA PLA_2s from other arthropods. PLA_2s are commonly

found throughout the animal kingdom where they are involved in various processes such as phospholipid digestion, rearrangement of cellular membrane phospholipid structures, inflammatory response, defence and predation mechanisms and signal transduction (Bowman et al., 1997). GMOY009713 has a signal peptide suggesting that the mature enzyme could be involved in the digestion of phospholipids in the extracellular space. Also its presence in the midgut of *G. m. morsitans*, an important site for the development and proliferation of trypanosomes, suggests that it could be involved in the defence of tsetse against trypanosome invasion, particularly during the early stage of the infection process.

Based on the possession of the features of kunitz-type serine proteinase inhibitors, which includes the presence of a low molecular weight kunitz domain consisting of conserved cysteine residues that form disulphide bonds and a P1 inhibitory site (Zupunski et al., 2003, Yuan et al., 2008), GMOY006016 is considered to belong to kunitz-type serine proteinase inhibitors. Kunitz-type serine proteinase inhibitors may be made up of a single domain, or a bis-domainal molecule containing two kunitz inhibitor-relaed domains or the domain may be repeated many times as in *Ancylostoma caninum* (hookworm) with 12 domains to form a multi-domain, single-chain inhibitor that can interact with several proteinase molecules (Ranasinghe and McManus, 2013). GMOY006016 contains a potential signal peptide as predicted by SignalP 4.1 and two kunitz domains with twelve cysteine residues forming six disulphide bonds and a putative P1 (Lys) inhibitory reactive site. The P1 residue in the inhibitory loop is the main determinant of the specificity of a particular kunitz protein and is occupied by lysine or arginine in most cases (MacLean et al., 2004).

Kunitz serine proteinase inhibitors in the saliva of blood feeding arthropods act as antihaemostatic factors preventing blood coagulation during feeding. The tick anticoagulant peptide (TAP) from the soft tick, *Ornithodoros moubata*, inhibits the formation of factor Xa (Waxman et al., 1990), the hard tick and Lyme disease vector, *Ixodes scapularis* has kunitz serine proteinase inhibitor which inhibits factor VIIa-tissue factor complex (Francischetti et al., 2002) and the black fly (*Simulium vittatum*) kunitz inhibitor which inhibits factor Xa, elastase and cathepsin G indicating its involvement in the regulation of both blood clotting and

inflammatory responses of the host (Tsujimoto et al., 2012). In blood feeding arthropods, kunitz-type serine proteinase inhibitors act as defence against microbial challenge in addition to their anticoagulant function. It has been shown that the stimulation of *Drosophila melanogaster* with bacteria or fungi leads to an up-regulation of two Kunitz proteins (De Gregorio et al., 2001). Also infection of *Rhipicephalus (Boophilus) microplus* with *Babesia bovis* led to an increase in the expression of a Kunitz proteinase inhibitor (Rachinsky et al., 2007) and a Kunitz-type molecule isolated from the tick *Dermacentor variabilis* has been shown to exhibit both bacteriostatic and anticoagulant properties (Ceraul et al., 2008).

3.4.2 Phylogenetic relationships

Eukaryotic cells possess the innate ability to detect and react to perturbations arising from abnormal development of cells and pathogen challenge (Nappi et al., 2004). Innate immunity as a mechanism of defence involves several elements such as cell signalling pathways, transcriptional elements and cytotoxic effector responses which are known to be conserved (Beutler, 2004). Comparative genetic and molecular studies have shown that processes that are involved in innate immune signalling pathways that culminate in the production of pathogen-specific cytotoxic responses by the innate immune systems are evolutionarily conserved (Nappi et al., 2004). A phylogenetic approach was therefore used to compare four candidate genes expressed in the midgut of self-cleared flies with counterparts from various other species.

For OGT, the greatest degree of homology was between tsetse and *Drosophila* while there was distant relationship between tsetse and other Dipterans. Tsetse CHT protein sequence showed high degree of similarity to other insects CHT as well mammals (*H. sapiens*). This could be as a result of the fact that chitinases are widely distributed in nature as they are found in species from all kingdoms performing different functions such as digestion, arthropod moulting, defence/immunity and pathogenicity (Arakane and Muthukrishnan, 2010). Phylogenetic analysis shows a high level of similarity between tsetse SPI and other invertebrate SPI especially *Drosophila*. SPI protein sequences from various species form three main phylogenetic groups. The tsetse (a blood

feeder) SPI showed significant homology with that of *Drosophila* (a non-blood feeder) as well as *Echinococcus granulosus* and *Ancylostoma ceylanicum* suggesting that serine proteinase inhibitors may not have evolved as a consequence of haematophagy and clustering may be by protein function.

3.4.3 Homology modelling

Homology modeling was used to develop 3D models for GMOY000153, GMOY002400, GMOY009713 and GMOY006016 (Figure 3.15). The biochemical functions of proteins can be deduced by their structures (Kleywegt and Jones, 1998). Proteins in their native surroundings adopt a unique 3D structure which in turn determines their biochemical functions (Floudas et al., 2006). The three-dimensional (3D) structure of a particular protein provides important information about its functions and interaction with other proteins and other compounds such as ligands (Tramontano, 1998). Homology or comparative modelling which is based on the observation that sequence similarity is equivalent to structural similarity remains the most accurate method to predict the three-dimensional structure of proteins (Lambert et al., 2002). It must be said however, that the quality of the predictions is dependent on the level of sequence similarity. More than 50% similarity between target and template represents a high quality prediction while significant errors would occur if the sequences share less than 30% identity (Floudas et al., 2006). In this study the lowest level of similarity obtained between a target protein and its template sequence was 36.9% for the CPHmodels server that was used in the prediction of the 3D models (Table 3.7). ModRefiner was used to improve the resolution of the predicted models, since most structural prediction algorithms aim to assemble structures as reduced models that represent amino acids by a reduced number of atoms in order to speed up the conformational search (Xu and Zhang, 2011). A refinement simulation step is therefore necessary if high-resolution models are to be obtained (Bradley et al., 2005). ModRefiner aim to draw the initial starting models closer to their native states in terms of hydrogen bonds, global topology and side-chain positioning (Xu and Zhang, 2011) and its use led to an improvement in the qualities of the models (Figure 3.15). Using COFACTOR,

putative binding sites and ligands were predicted for the candidate genes. Chitinase was predicted to contain multiple ligands and 15 putative binding sites while OGT contains 8 putative binding sites and a uridine-5′-diphosphate (UDP) ligand which is an important factor in glycogenesis. PLA_2 contains 9 putative binding site residues while SPI contains peptide ligand and 9 putative binding site residues. Prediction of protein-ligand binding sites is very important in determining the functions of proteins since in most cellular processes, proteins interact with other molecules to perform their biological functions (Dai et al., 2011).

3.4.4 Summary

In this chapter it has been shown that phylogenetically, the genes that are differentially expressed in the midgut of refractory flies share sequence homology with genes involved in immune response in other insects. Also tsetse flies react to infection by trypanosomes by differentially expressing some set of genes as revealed by the analysis of the transcriptome data from the midgut of uninfected flies versus self-cleared flies. Additionally, we also used sequence analysis to reveal certain conserved motifs that are present in the genes that are essential for their functions. Finally we also used homology modeling to determine the 3D structures of the genes.

CHAPTER 4

Validating the role of immune related genes *CHT, OGT and SPI* in the midgut of trypanosome-refractory flies using RNAi silencing

4.1 Introduction

Insects are very often exposed to different types of parasites, but because they possess a complex and very effective immune system, they succeed in eliminating the invading parasites in most cases resulting in very few of the insects developing infection (Barribeau and Schmid-Hempel, 2013, Gillespie et al., 1997).

In this chapter, RNAi knockdown of three immune related genes (chitinase, GMOY000153, *O*-GlcNAc transferase, GMOY002400 and serine proteinase inhibitor, GMOY006016) that were differentially regulated in the midgut of flies that cleared the parasites after being challenged with *T. brucei* was carried out. This was done to find out what effect the loss of the functions of these genes will have on the susceptibility of tsetse to trypanosome infection. These genes were chosen based on the important roles they play in the immunity of arthropods. The tsetse refractory phenotype observed in the field can be reproduced in the lab by feeding flies with normal blood prior to receiving an infected blood meal. These conditions were used to produce refractory flies and the genes of interest were down-regulated using RNAi before supplying the infective blood meal. Flies were dissected and scored for infection 7 days post-infection.

4.2 Materials and methods

The materials and methods are as outlined in chapter 2

4.3 Results

4.3.1 Effect of RNAi knockdown of genes by injection of dsRNA

RNA interference (RNAi) was used to knockdown the expression of tsetse chitinase (GMOY000153), OGT (GMOY002400) and SPI (GMOY006016) by injection of dsRNA. Flies were injected with 5 µg, 7 µg and 10 µg of dsRNA to determine the optimal concentration of dsRNA needed to achieve maximum knockdown without causing much mortality. The injection of 10 µg of dsRNA achieved significant knockdown of 68%, 62% and 69% respectively for GMOY000153, GMOY002400 and GMOY006016 respectively (Figure 4.1) and minimal mortality rates of 8%, 7% and 10% respectively (Table 4.1). The injection of dsRNA-GFP was used as control and produced no significant knockdown effect over the same period (Figure 4.1). This demonstrates that the knockdown effect observed was not as a result of injecting, but rather the effect of the injected dsRNA on the target genes.

Table 4.1 Mortality rates observed in flies after injection with different concentrations of dsRNA of genes of interest. Flies (n=45) were injected with 5 µg, 7 µg and 10 µg of dsRNA. Three replicates were used and data are presented as mean value ± SE.

	Mortality rate		
	CHIT	***OGT***	***SPI***
5 µg/fly	5%	6%	5%
7 µg/fly	7%	10%	8%
10 µg/fly	8%	7%	10%

Table 4.2 Transcript knockdown in midgut following injection with different concentrations of dsRNA of genes of interest. Flies (n= 45) were injected with 5 µg, 7 µg and 10 µg of dsRNA. Three replicates were used and data are presented as mean value ± SE.

	Transcript knockdown		
	CHIT	***OGT***	***SPI***
5 µg/fly	38%	43%	35%
7 µg/fly	35%	47%	44%
10 µg/fly	68%	62%	69%

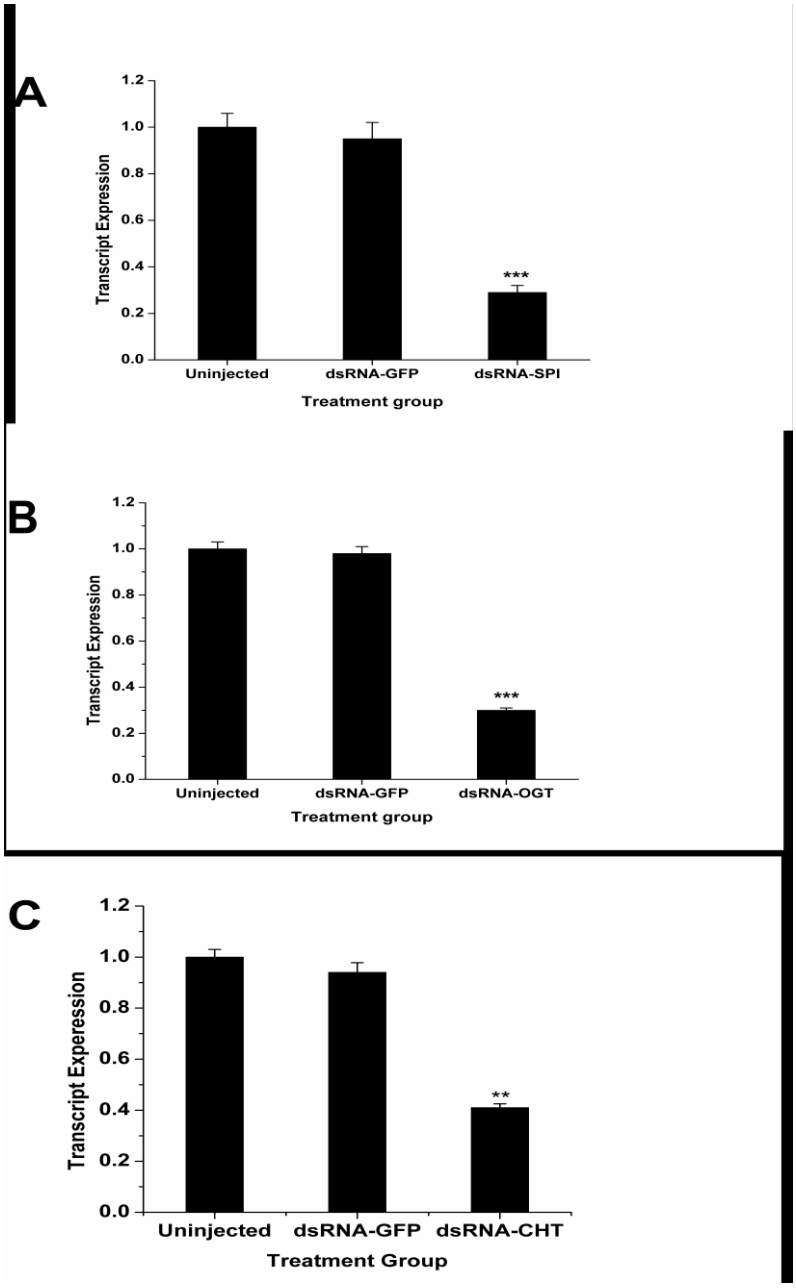

Figure 4.1 Transcript knockdown of Cht, OGT, PLA_2 and SPI following dsRNA injections. QPCR analysis of midgut tissues of flies injected with 10µg of dsRNA-SPI, dsRNA-OGT, and dsRNA*CHIT* and infected with trypanosomes. Midgut tissues were analyzed for transcript expression 7 days after infection. Transcript levels of SPI, OGT and CHT were decreased approximately 68%, 62% and 69% respectively compared to controls (uninjected and dsRNA-GFP-injected). p-values- (**) = 0.001; (***) = 0.0001. Data are presented as mean value of three replicates (45 flies per replicate) ± SE.

4.3.2 Survival rate of flies following injection of dsRNA.

To monitor the effect of the injection of dsRNA on the longevity of the flies after the injection of dsRNA, flies were monitored every 5 days to see if the wound sustained as a result of dsRNA injection led to unusual death of the flies. The flies were fed normal defibrinated horse blood on the day of emergence. They were injected with dsRNA of the genes of interest the following day, fed a normal blood meal the next day, left to rest for a day, and then infected with *T. b. brucei* TSW196 bloodstream form parasites and then observed for occurrence of death during the course of the experiment and beyond. The observation was extended beyond the duration of the experiment to see if there was any latent effect that may have affected the health of the flies but was not manifest during the time the experiment lasted. The results show that there were no significant differences between flies injected with dsRNA compared with uninjected control during the course of the experiment (Figure 4.2). This demonstrates that the injection of dsRNA did not weaken the flies as to affect their immune response.

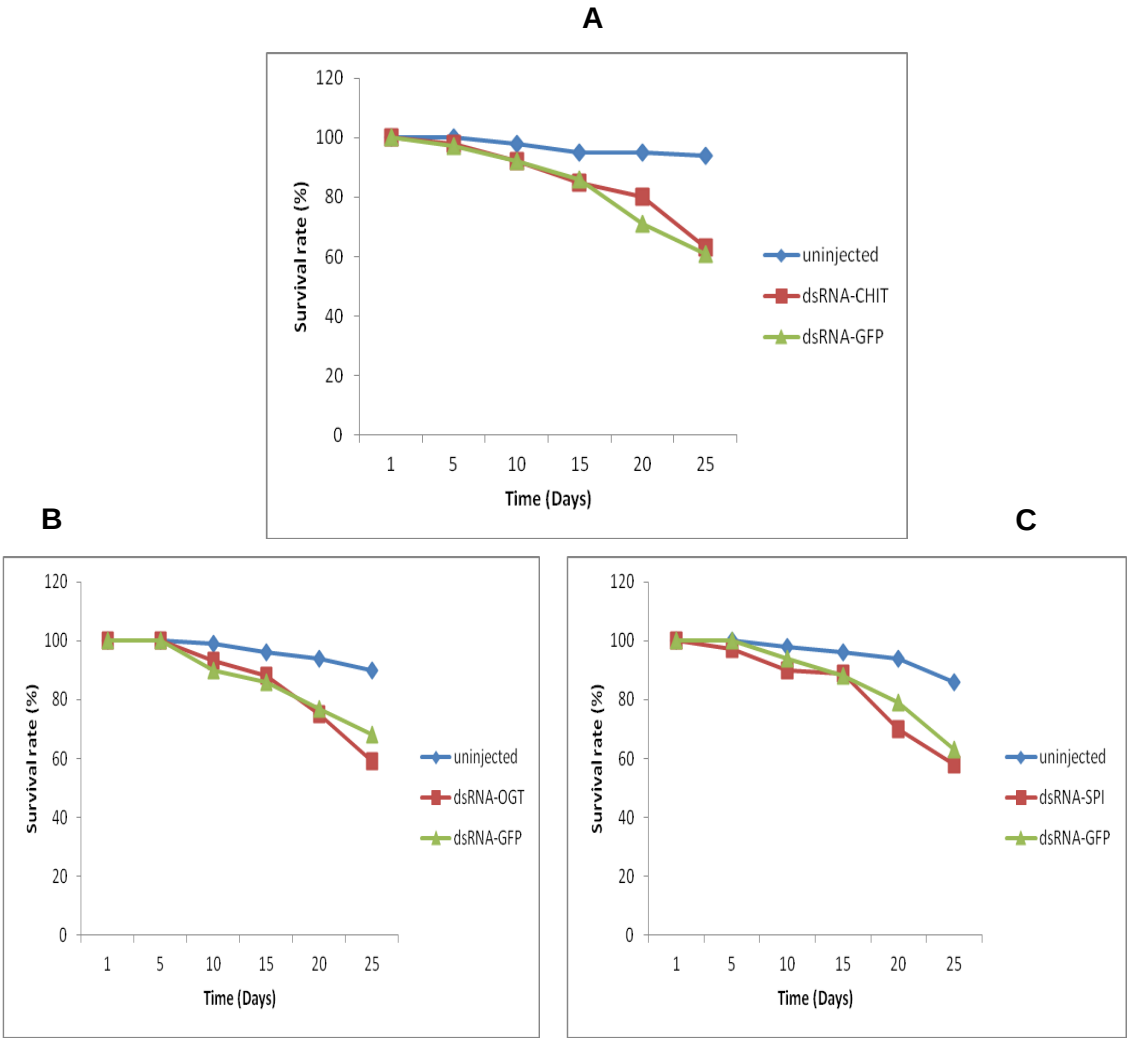

Figure 4.2 Survival rates of flies challenged with trypanosomes after dsRNA injection. Flies (n=45) were fed normal defibrinated horse blood on the day of emergence and then injected with 10 µg dsRNA of genes of interest 24 hours later. The survival rates of flies were then monitored during the course of the experiment to check the effect of injecting dsRNA on the longevity and fitness of the flies. **The observation was extended beyond the duration of the experiment to see if there were latent effects that may have affected the health of the flies but was not manifest during the time the experiment lasted.** There were no significant differences between flies injected with dsRNA compared with uninjected controls during the course of the experiment. Data are presented as mean value for three replicates ± SE.

4.3.3 Trypanosome infection prevalence after dsRNA injection

In order to determine the function of the genes of interest in relation to trypanosome infection, the expression of each gene was suppressed using RNAi and subsequently infected with trypanosomes. Then flies were dissected to check for midgut infection. Flies were fed normal blood meal on the day of emergence and were injected with 10 µg of dsRNA the following day. Flies were infected at the third blood meal and dissected 7 days post-infection to check for midgut infection. Trypanosome infection prevalence in flies injected with dsRNA increased significantly compared with the two controls (uninjected and dsRNA-GFP treated flies) (Figure 4.3). In flies injected with dsSPI there was a 41% increase in trypanosome infection prevalence compared to uninjected and dsRNA-GFP-injected controls (Figure 4.3A); flies injected with dsOGT had 38% increase in trypanosome infection rate compared to uninjected and dsRNA-GFP-injected controls (Figure 4.3B); while in flies injected with dsCHIT there was a 31% increase in trypanosome infection rate compared to uninjected and dsRNA-GFP-injected controls (Figure 4.3C). Investigating the effect of injecting dsRNA on the mortality of the flies showed no significant increase in mortality rate compared to uninjected control.

When tsetse flies are infected after having a couple of normal blood meals, trypanosome infection prevalence is usually less than 10% (Haines et al., 2010). In this experiment, flies were fed normal blood meal after emergence before being injected with dsRNA, followed by another blood meal and the third blood meal was contained *T. b. brucei* TSW196. Dissecting the flies after 7 days to check for midgut infection revealed significant increase in infection rate in flies injected with dsRNA of genes of interest while infection prevalence in the control groups were consistent with the normal rates of infection observed in flies that had a couple of normal blood meals prior to the infectious blood meal (less than 10%).

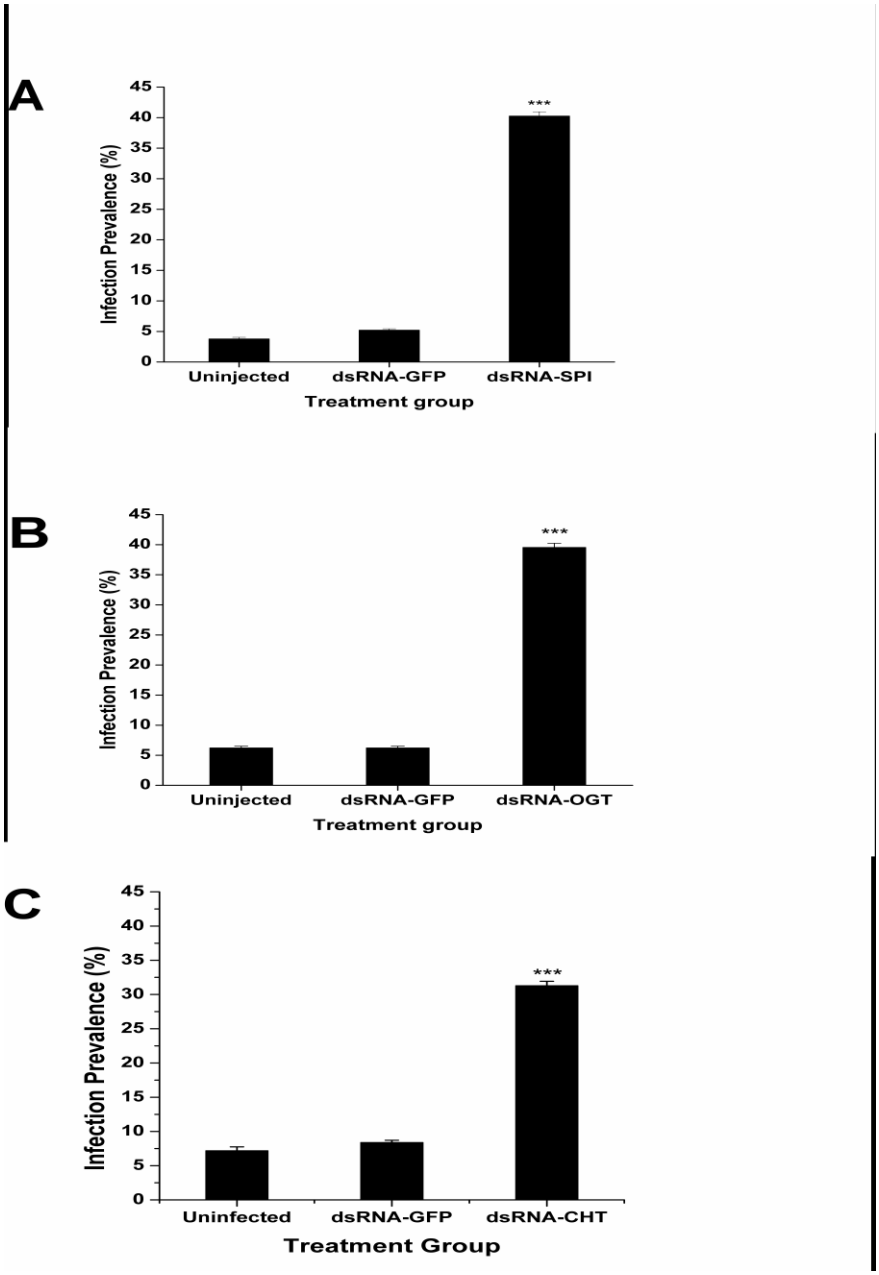

Figure 4.3 Trypanosome infection prevalence following RNAi knockdown. Trypanosome infection prevalence following RNAi knockdown of *SPI*, *OGT* and *CHT*. Flies (n=45) were fed normal bloodmeal on the day of emergence, injected with dsRNA the following day (10 µg/fly) and then fed infectious bloodmeal (3rd bloodmeal) and dissected 7 days later. The infection prevalence values are presented for the controls (uninjected and dsRNA-GFP-injected) and dsRNA-SPI, dsRNA-OGT and dsRNA-CHT-treated groups. RNAi knockdown of candidate genes resulted in significant increase ($p = 0.0001$) in infection prevalence for dsRNA-SPI, dsRNA-OGT, and dsRNA-CHT-treated flies respectively compared with controls (uninjected and dsRNA-GFP). There was no significant difference in infection prevalence between the

controls (uninjected and dsRNA-GFP-injected flies). Data are presented as mean value for three replicates ± SE.

4.4 Discussion

RNA-mediated interference or RNAi for short is a valuable tool for the investigation of the functions of a gene *in vivo*. With RNAi one can knockdown the expression of a gene and then observe the loss-of-function phenotypes afterwards. Using RNAi, any gene whose sequence is known can be targeted using the dsRNA of a sequence that is identical to the target gene. By introducing the dsRNA of the homologous sequence into the organism or cell, the dsRNA is cut by Dicer into short interfering RNAs (siRNA) of approximately 21-23 bp which is involved in the silencing mechanism (Carthew and Sontheimer, 2009), after associating with an RNA-induced silencing complex (RISC) leading to the degradation of the target gene mRNA by cleaving the target mRNA in the middle of the complimentary region (Meister and Tuschl 2004).

In this chapter the involvement of three immune related candidate genes (*CHT, OGT and SPI*) that were differentially expressed in the midgut of refractory flies in the control of infection were investigated by using RNAi. The midgut of the fly is an important site for the establishment of trypanosome infection. For the parasites to have a chance of completing their life cycle in the fly, they must first of all establish infection in the midgut. It is aslo a very hostile environment for the development of the parasites because the fly mounts a robust immune reaction in its bid to get rid of the parasites. For the fact that these genes were differentially expressed in the midgut of flies that were resistant to trypanosome infection compared to flies that were not challenged by trypanosomes suggests that they could be involved in the process of elimination of infection. Also since the differential expression of these genes were observed to occur during the period when the fly tries to eliminate the invading parasites (the first 3 days after trypanosome challenge), a period characterised by a process of attrition that leads to the complete elimination of infection in some flies (Gibson and Bailey, 2003).

Chitin and chitin-likeproteins are among the main features of the invertebrate and vertebrate immune systems (Beckerman et al., 2013). The regulation of chitin synthesis and degradation is thought to be central to arthropod defences (Beckerman et al., 2015). The pritrophic matrix of arthropod guts which forms a barrier and a major line of defence against pathogens and parasites is rich in chitin (Dinglasan et al., 2009). Also, some features such as the phenoloxidase (PO) cascade, melanization and antimicrobial peptides which are heavily involved in the arthropod innate immune system have been linked to chitin metabolism (Beckerman et al., 2013). Many antimicrobial peptides are chitin-binding and are therefore thightly linked with chitinases (Tran et al., 2011). Even the PO cascade is thought to be triggered by chitin-binding antimicrobial peptides (Nagai et al., 2001). Also melanin, a major feature of invertebrate immune system is thought to be regulated by chitin metabolism (Marmaras et al., 1996, Walker et al., 2010). Therefore, in arthropods, the immune system can be said to be highly dependent on the regulation of chitin synthesis and degradation. In arthropods, the midgut is of strategic importance because it is the point of entry for many arthropod parasites and it is not surprising therefore that it acts as a barrier to parasites both structurally and immunologically forming a first line of defence in the fight against parasite invasion.

Since chitinase plays an important role in arthropod immunity, it therefore follows that its inhibition could impair immune reactions against invading parasites. In order to check if the inhibition of chitinase in tsetse will result in increase in infection rate, we carried out RNAi knockdown of chitinase and subsequently challenged the flies with trypanosomes. There was a significant increase in midgut infection in flies injedted with dsRNA-CHT compared to unjected and dsRNA-GFP-injected controls. This suggests that the knockdown of chitinase in tseste paves the way for trypanosome midgut establishment. The inhibition of chitinase could have a negative impact on the defensive roles of the peritrophic matrix since chitinase is involved the regulation of chitin (a major component of the peritrophic membrane) synthesis and degradation. It is therefore possible that the inhibition of chitinase could perturb the peritrophic matrix function in infected tsetse, thereby leading to increased susceptibility to trypanosome infection. The role of chitinase in the formation of the PM and involvement in the

modulation of immune response in tsetse can be investigated by supplementing the bloodmeal with a known chitinase inhibitor such as allosaminidin and measuring the susceptibility of the flies to trypanosome infection compared to trypanosome challenged flies that did not receive allosaminidin.

Invertebrates rely solely on their innate immune system for defence against invading pathogens (Engelmann and Pujol, 2010). The use of O-GlcNAc transferase (OGT) as an immune modulator is evolutionarily conserved in both vertebrates and invertebrates (Bond et al., 2014). In *Caenorhabditis elegans*, OGT acts through the β-catenin (BAR-1) pathway together with p38 MAPK (PMK-1) to modulate immune response to *Staphylococcus aureus*, and mutant animals displayed a deregulation of stress-and immune-responsive genes in addition to being hypersensitive to *S. aureus* (Bond et al., 2014). OGT catalyses the addition of O-linked N-acetylglucosamine (O-GlcNAc) to serine and threonine residues (Hanover et al., 2010). This post-translational modification (PTM) of proteins plays a vital role in numerous cellular signalling pathways involving growth, metabolism, cellular stress and host-pathogen interactions (Hanover et al., 2010). The presence or absence of PTM can have a profound effect on protein properties such as enzyme activity or regulation (Du et al., 2001, Federici et al., 2002, Parker et al., 2003), localisation and acivity (Juang et al., 2002). There is the notion that inappropriate O-GlcNAcylation of specific, key OGT targets brought about by the absence of OGT will lead to immunodeficiency (Bond et al., 2014).

To check if this notion holds true for tsetse, OGT expression was suppressed using RNAi and flies were subsequently challenged with *T. b. brucei* to determine whether this will lead to increased midgut infection. A significant increase in midgut infection was observed in flies injected with dsRNA-OGT compared with uninjected control and dsRNA-GFP-injected cotrols. This suggests that the inhibition of OGT expression resulted in increased susceptibility of flies to trypanosome infection. From this result it can be concluded that OGT is essential for immune response in tsetse. It is possible that in the absence of OGT, which means no O-GlcNAcylation, proteins lacking O-GlcNAc may be inappropriately expressed, localised, or activated leading to a poorly cordinated immune response. I therefore suggest that OGT is essential

for tsetse immune response and plays a vital role in the tsetse innate immune response to trypanosome infection and may be a key component of its immune regulation systemsince its suppression using RNAi led to a significant increase in trypanosome infection rate.

Insects possess an innate immune system that is made up of both humoral and cellular responses which operate in a coordinated way when reacting to infection by pathogens or parasites (Franssens et al., 2008). The serine proteinase inhibitors in arthropods are thought to protect their hosts from infection by inhibiting proteinases from pathogens or parasites in addition to regulating endogenous proteinases that are involved in coagulation, prophenol oxidase (PPO) activation or cytokine activation (Kanost, 1999). Pathogens possess proteinases that help them to penetrate the cuticle of arthropod hosts and can aslo contribute to the virulence of bacterial pathogens (Kanost, 1999). However, some proteinase inhibitors in arthropods may play a role in defending the host against such microbial proteinases as has been demonstrated in *Bombyx mori* in which a fungal proteinase inhibitor (FPI) is known to be active against proteinases from fungal pathogens (Eguchi et al., 1993). Also several variants of the *Manduca sexta* serpin-1 gene are known to inhibit bacterial or fungal serine proteinases (Jiang and Kanost, 1997).

To obtain more information about the role of SPI (GMOY006016) in tsetse, RNAi targeting GMOY006016 was induced in tsetse followed by infection with *T. b. brucei*. The significant increase in trypanosome infection rate following the knockdown of GMOY006016, coupled with the fact that it is secreted in the midgut where it is in contact with invading trypanosomes suggests that it is involved in immune defences against trypanosome infection. An important component of the humoral immune response in arthropods is the prophenoloxidase-activating system (proPO-AS) (Cerenius and Soderhall, 2004, Kanost et al., 2004). When pathogens succeed in penetrating the cuticular barrier, a second line of immune defense reactions is induced leading to the limited activation of the prophenoloxidase (proPO) precursor into the active phenoloxidase (PO), culminating in the production of melanin and associated toxic intermediary compounds which kill invading pathogens (Franssens et al., 2008). However, the activation of the proPO must be tightly controlled to avoid

premature activaion and to prevent it from acting beyond the inteded target. This is partly achieved by the presence of serine proteinase inhibitors (SPI) which prevent unwanted activation of this complex which have the potential to cause undesirable destructive action if allowed to act beyond their intended target. We observed a significant increase in trypanosome infection in the midgut of dsRNA-SPI knockdown flies compared to uninjected and dsRNA-GFP-injected flies (Figure 4.3 A) suggesting that SPI is involved in tsetse immune response during trypanosome infection. In the event of the inhibition of the fly's SPI, uncontrolled activation of the proPO might occur which will have deleterious effect on the fly thereby affecting its ability to fight trypanosome infection because the quinones and reactive oxygen species generated by uncontrolled spread of PPO activation would be harmful to the fly (Zhu et al., 2003). The effect of uncontrolled activation of PPO on the fitness of the fly can be tested by inhibiting the genes that regulate the production such serine proteinase inhibitor and then observe the survival rate of the flies compared controls. It is also possible that the inhibition of GMOY006016 affected the proper coordination of the activation of the proPO-AS which may lead to untimely activation of the proPO and which may impart negatively on the fly's immune response to trypanosome infection, hence the increased susceptibility of the flies to trypanosome infection.

To further validate the roles of these genes in tsetse immune rseponse, it will be good to examine potential interactions between the candidate genes to uncover potential pathways involved in tsetse refractoriness. Also it will be interesting to knock down two or more candidate genes simultaneously and observe its effect on tsetse in relation to susceptibility to trypanosome infection.

Chapter 5

Role of sPLA$_2$ (GMOY009713) in the establishment of midgut infection in *Glossina morsitans morsitans*

5.1 Introduction

Phospholipases A_2 (PLA_2s) are a family of enzymes that catalyze the hydrolysis of the second carbon group of the glycerol moiety of glycerophospholipids to release (mostly unsaturated) fatty acids and lysophospholipids (Murakami et al., 2011b). They are present in viruses, bacteria, plants and animals (Farr et al., 2005, Sato and Frank, 2004, Fujikawa et al., 2005, Six and Dennis, 2000, Valentin and Lambeau, 2000). Secreted PLA_2s are the most abundant in the PLA_2 family making up about one third of all PLA_2s and are characterised by low molecular weight, require Ca^{2+}, are secreted and possess a His/Asp catalytic dyad (Murakami et al., 2011b). They are synthesized by many different cell types, and are secreted in various body fluids where they are involved in a variety of pathophysiological processes such as the breakdown of dietary phospholipids, remodelling of cellular membrane, signal transduction, production of lipid mediators, inflammatory reaction, host immune defences and venom toxicity (Stanley, 2006a, Nevalainen et al., 2000, Nevalainen et al., 2008, Fry et al., 2009, Murakami et al., 2010, Murakami et al., 2011a). They can also act as ligands in many biological processes that mediate several pathological and biological interactions among organisms (Lambeau and Lazdunski, 1999, Hanasaki and Arita, 1999, Rouault et al., 2003, Triggiani et al., 2005).

In insects, sPLA$_2$s activities are important for the digestion of dietary phospholipids (PLs) helping them to meet the essential dietary requirements for polyunsaturated fatty acids by hydrolysing the *sn-2* fatty acids, which are then absorbed via the midgut epithelium (Stanley, 2006a). Also since insects, unlike vertebrates do not produce bile salts whose function is to solubilise lipids and make them available for digestion and subsequent absorption, the lysophospholipids liberated from phospholipids by the catalytic activity of PLA_2 perform the function of bile salts by acting as solubilisers thereby facilitating lipid

digestion (Stanley, 2006a). It has been suggested that $sPLA_2$s are a common and important feature of the digestive physiology of insects (Uscian et al., 1995).

It has been suggested that prostaglandins (PGs) and other eicosanoids play important role in insect cellular immunity (Jurenka et al., 1999). Also, it has been established that in the event of microbial challenge, PLA_2 activity increases in insects (Yajima et al., 2003, Tunaz et al., 2003, Shrestha and Kim, 2007, Shrestha and Kim, 2010). The biosynthesis of eicosanoids begins with the hydrolysis of arachidonic acid (AA) leading to the release of lipid mediators such as prostaglandins, thromboxanes and leukotrienes (Dennis et al., 2011). The enzymatic action of PLA_2 on phospholipids with the release of AA is therefore the first and rate-limiting step for the biosynthesis of eicosanoids (Dennis et al., 2011). It has been hypothesised that eicosanoids play an important role in insect immune responses when challenged by bacteria (Stanley-Samuelson et al., 1991, Miller et al., 1994). Eicosanoids-mediated actions in insects include microaggregation and nodulation reactions to bacterial infections, prophenoloxidase (PPO) activation, phagocytosis and cell spreading (Miller et al., 1994, Downer et al., 1997, Miller, 2005). Inhibition of PLA_2 led to significant reduction of phagocytosis of protozoan parasites (Figueiredo et al., 2008b) and prevented encapsulation of parasitoid eggs (Carton et al., 2002).

It has been proposed that the eicosanoid signaling system is involved in a cross-talk with plasmatocyte-spreading peptide (PSP) (Srikanth et al., 2011), an insect cytokine that mediates plasmatocytes spreading (Clark et al., 1998). Its precursor protein, the proPSP is expressed by granular haemocytes which subsequently release the PSP which rapidly adhere and spread at the target following the subjection an insect to immune challenge (Eleftherianos et al., 2009). The initiation of infection by pathogens provokes the production of activated PSP which in turn activates PLA_2 leading to the biosynthesis of prostaglandins with the ultimate effect of plasmatocytes spreading (Srikanth et al., 2011). This again establishes eicosanoids, and invariably PLA_2 as an important element of the immune signaling system in insects. Perhaps, the most important indication that PLA_2 plays an important role in insect immune responses is the fact that certain microbial pathogens produce and secrete

compounds that disable insect immune responses by specifically inhibiting PLA_2 (Stanley, 2006a). These pathogens exert their influence on host insects by blocking the immune-mediating eicosanoid pathway by their inhibitory action on the first step in eicosanoid biosynthesis catalysed by PLA_2 (Park and Kim, 2000). For example, the entomopathogenic nematode, *Steinernema carpocapsae*, a mutualistic symbiont with the bacterium, *Xenorhabdus nematophila,* after invading an insect host is known to release *X. nematophila* (a member of the Enterobacteriaceae family) into the haemolymph. The bacterium is thought to inhibit the insect's immune reactions to the invading nematode by secreting substances that directly inhibit $sPLA_2$ and also proliferates rapidly after being released and kills the insect host with the insect cadaver serving as a conducive microhabitat suitable for the nematode to complete its development providing nutrients and protection from other microbes (Park and Kim, 2000). Bacterial factors are known to be potent inhibitors of $sPLA_2$ from different sources (Park et al., 2004). Another bacterium in the Enterobacteriaceae family, *Photorhabdus temperate*, is known to similarly inhibit PLA_2 in insects (Kim et al., 2005). It may therefore be possible that other bacterial species in this family may also exhibit the same property of impairing insect immune responses by inhibiting $sPLA_2$. In mammals, Gram-positive bacteria are thought to protect themselves from the bactericidal action of $sPLA_2$ by secreting toxins which reduce the amount of PLA_2 rather than inhibiting the activity of PLA_2 as seen in insects (Gimenez et al., 2004).

Apart from bacteria, protozoan parasites are also known to impair host immunity through the inhibition of eicosanoid biosynthesis. Experiments with *Rhodnius prolixus* shows that oral infection with *Trypanosoma rangeli* leads to suppression of eicosanoid biosynthesis just as the addition of dexamethasone, an indirect inhibitor of PLA_2 to the blood meal of *R. prolixus* inhibited phagocytosis (Garcia et al., 2004). This inhibitory effect of dexamethasone was reversed when the larvae are treated with AA or with platelet activating factor (Figueiredo et al., 2008b). The fact that some microbial pathogen aim to suppress host immune responses by inhibiting PLA_2, a key enzyme in eicosanoid biosynthesis, points to the fact that PLA_2s play important roles in insect immune responses.

G. morsitants expresses a $sPLA_2$ activity in both the midgut and the salivary glands (see characteristics of this protein in Chapter 3), which is slightly down-regulated in self-cured flies that have previously received a trypanosome-infected blood meal. In this Chapter I describe the pattern of secretion of this sPLA2 during a trypanosome infection and show evidence that this activity may have a role in controlling the levels of parasite infection in the tsetse midgut.

5.2 Materials and methods

Materials and methods are as outlined in section 2.

5.3 Results

5.3.1 Tsetse PLA_2 mRNA and protein levels were significantly reduced following gene knockdown by RNA interference

RNAi-mediated knockdown was used to validate the role of *Gmm* $sPLA_2$ in controlling the establishment of a trypanosome infection in the tsetse midgut. Injection of dsRNA (as described in section 2.5) resulted in a significant (~72%) reduction in transcript level of $sPLA_2$ compared to controls (Figure 5.1). Similar results were obtained by western blotting, which revealed elimination of the endogenous protein in midguts of flies following the injection of 10 µg of dsRNA-$sPLA_2$ (Figure 5.2). Corroboration of the protein expression after RNAi silencing is important as using only transcript levels can sometimes be misleading for some genes (Gygi et al., 1999), particularly in haematophagous insects where post-transcriptional regulation is a common feature (Hamilton et al., 2002). The injection of dsRNA-$sPLA_2$ therefore resulted in a significant reduction of both transcript expression and protein expression.

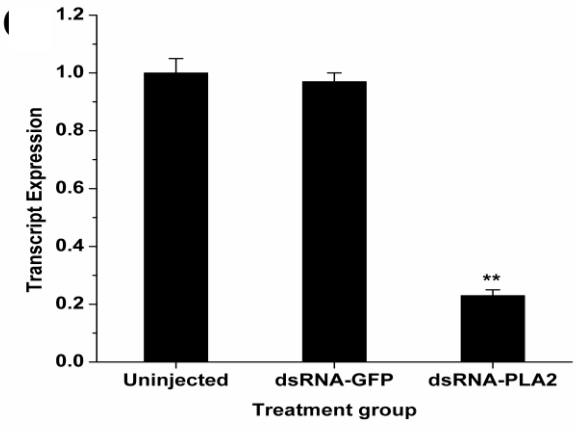

Figure 5.1 Levels of $sPLA_2$ expression after RNAi knockdown. dsRNA-$sPLA_2$ (10 µg per fly) was injected into adult flies. The knockdown level of PLA_2 transcripts was analysed by qPCR using cDNAs prepared from total RNA isolated from pools of five day 7 flies. Expression levels of $sPLA_2$ were presented relative to

the level in dsRNA-*GFP*-injected and uninjected control flies. The transcript levels of *G. m. morsitans* α tubulin and *β tubulin* were measured to normalize for differences in the concentration of cDNA templates between samples. Transcript levels of $sPLA_2$ were decreased approximately 72%. An asterisk indicates a significant difference in transcript levels between control and test flies (** = 0.001; *** = 0.0001). Data are shown as mean value ± SE (n= 3).

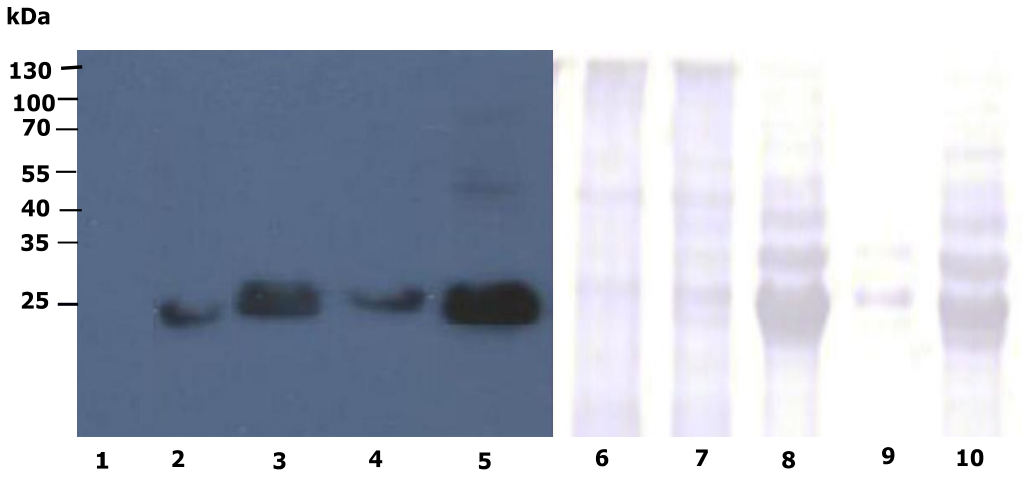

Figure 5.2 Tsetse PLA2 protein level after dsRNA injection. Flies were injected with 10 µg of dsRNA-*sPLA$_2$* 24 hours after receiving a normal blood meal and dissected after seven days. Five midguts were pooled for each sample for protein isolation which was used for western blot analysis. Lane 1: dsRNA-PLA_2 knockdown midguts; lane 2: dsRNA-GFP injected midguts; lane 3: unfed salivary glands (SG) lysate (positive control for $sPLA_2$ recognition); lane 4: Uninfected midguts; lane 5: SG (from fed flies). There was a decline in tsetse $sPLA_2$ protein levels following dsRNA-PLA_2 injection. The nigrosine-stained PVDF (6-10) is placed adjacent the immunoblot film.

5.3.2 Trypanosome infection prevalence is increased by RNAi-mediated knockdown of tsetse $sPLA_2$

To determine if $sPLA_2$ influences trypanosome establishment in the midgut of tsetse, flies were injected with dsRNA-$sPLA_2$ and subsequently offered an infectious blood meal. Typically, flies were allowed to recover for 24-48 hours after injecting dsRNA. They were then offered an infectious blood meal containing *T. b. brucei* (TSW196). Seven days after the infectious blood meal the flies were dissected and examined microscopically to check for midgut

infection. A statistically significant increase in susceptibility to trypanosome infection was observed in flies injected with dsRNA compared to the controls (Figure 5.3).

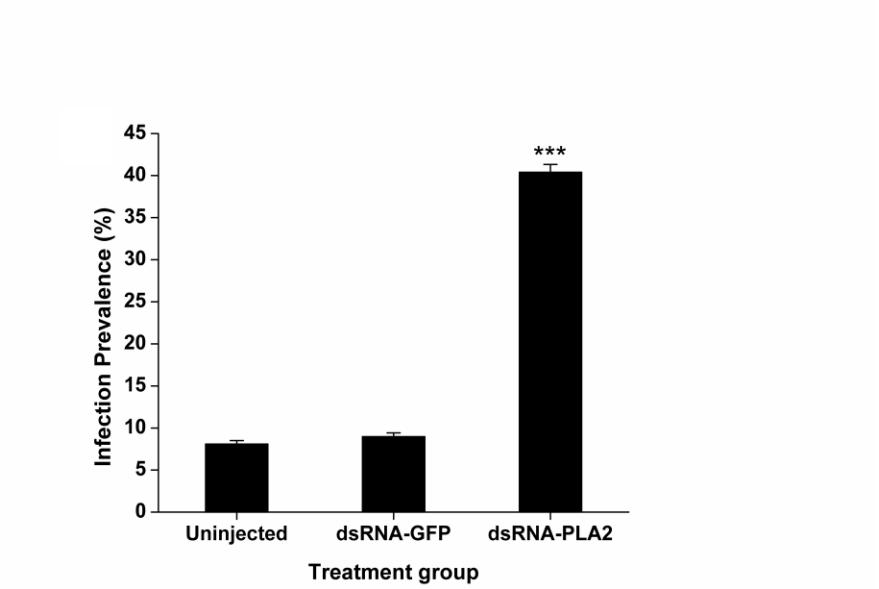

Figure 5.3 Trypanosome infection prevalence after dsRNA-PLA_2 injection. Trypanosome infection in the midgut of flies injected with dsRNA-PLA_2. Flies (n=48) were injected with 10 µg dsRNA-$sPLA_2$ and infected with BSF *T. b. brucei*. At 7 days post-infection, flies were dissected and the midguts analyzed microscopically to score for infection. RNAi knockdown of candidate genes resulted in significant increase ($p = 0.0001$) in infection prevalence for dsRNA-PLA_2-treated flies compared with controls (uninjected and dsRNA-GFP-injected flies). There was no significant difference in infection prevalence between the controls (uninjected and dsRNA-GFP-treated flies). Data are presented as mean value of three biological replicates ± SE.

5.3.3 Tsetse $sPLA_2$ expression decreased following infection with *T. b. brucei* bloodstream form (BSF)

Some bacteria and protozoans in a bid to suppress the insect host's immune response produce and secrete compounds specifically aimed at PLA_2 (Stanley and Kim, 2011). The microbes try to suppress the eicosanoid-mediated immune response through the inhibition of $sPLA_2$. To investigate if this is also true for tsetse, flies were infected with *T. b. brucei* BSF and the pattern of midgut expression of PLA_2 was monitored over 14 day period. Typically, flies were

infected in their first blood meal and maintained on normal blood meal every 48 hours for a period of 14 days. Dissections were carried out 24 hours after infection and at 3, 7 and 14 days post infection to extract midgut tissue from infected flies which were analysed for $sPLA_2$ transcript expression using qPCR. Initially, there was a suppression of $sPLA_2$ expression following trypanosome infection until day 14 post infection when a sharp increase in PLA_2 was observed compared to uninfected control over the same period (Figure 5.4 A). Similar results were obtained when the protein fractions from the same samples were analysed by western blotting using a commercial anti-PLA_2 antibody (Figure 5.4 B).

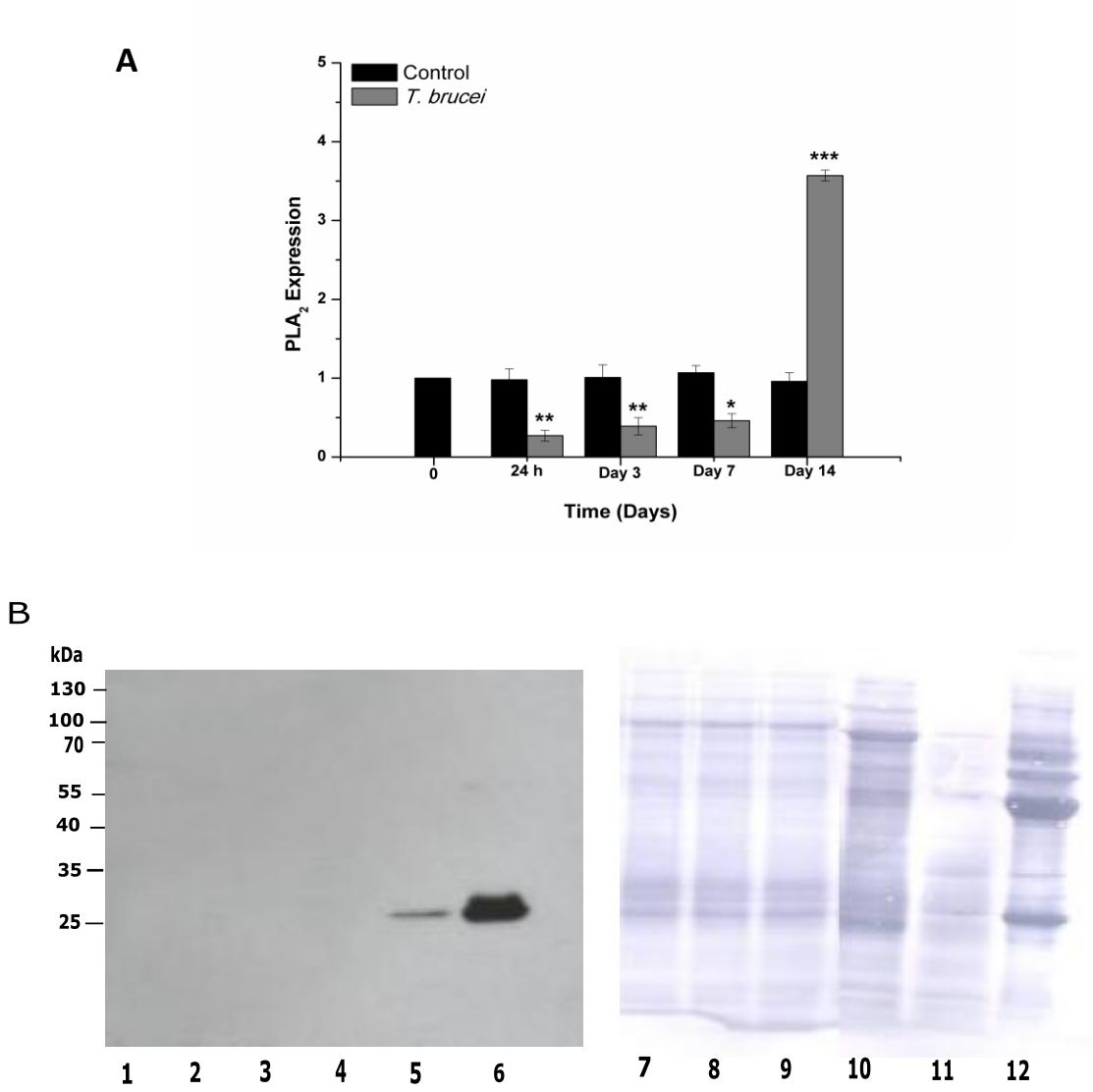

Figure 5.4 Timecourse of $sPLA_2$ expression after infection with *T. b. brucei* BSFs. (A) Analysis of $sPLA_2$ transcript expression using qPCR. Teneral flies (n=50) were infected with *T. b. brucei* and dissected 24 hours after infection and subsequently on days 3, 7 and 14 post-infection. Midguts were collected and analysed for *$sPLA_2$* transcript expression using qPCR. Expression levels of PLA_2 were presented relative to the level in uninfected controls. The transcript levels of *G. m. morsitans* α tubulin and *β-tubulin* were measured to normalize for differences in the concentration of cDNA templates between samples. Level of significance between test samples and control is indicated by asterisks, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data are shown as mean value ± SE (n = 3). (B) Left panel shows western blot analysis showing significant reduction in $sPLA_2$ protein levels between days 1 and 7 and an increase in PLA_2 protein level on day 14. Lane 1: day 0; lane 2: 24 hours: lane 3: day 3: lane 4: day 7: lane 5: day 14: lane 6: tsetse SG extract (positive

control). The nigrosine-stained PVDF membrane (7-12) is placed adjacent to the immunoblot film (right panel).

5.3.4 Infecting flies with procyclic forms (PCF) of *T. b. brucei* does not alter the $sPLA_2$ expression profile

Since there was an increase in the expression of $sPLA_2$ on day 14 following the infection of tsetse with blood stream forms of *T. b. brucei*, we thought the suden increase in the expression of PLA_2 on day 14 after trypanosome infection could be related to an increase in the number of procyclic forms. We also wanted to know if there will be a drop in the exporession of $sPLA_2$ beyond day 14. We therefore decided to infect flies directly with procyclic forms of *T. b. brucei* and also extended the time of infection up to 15 days. Teneral flies were fed infectious blood meal containing 1×10^5 cells/mL and 1×10^6 cells/mL of procyclic forms. Flies were dissected at the same time points used for blood stream form experiment to extract midgut tissues which were subsequently analysed for PLA_2 expression.

The infection of flies with procyclic trypanosomes resulted in an initial suppression of PLA_2 expression following trypanosome infection until day 15 post infection when a notable increase in $sPLA_2$ was observed compared to uninfected control over the same period. Furthermore, feeding flies different parasite loads (1×10^5 cells/mL and 1×10^6 cells/mL) did not alter the expression pattern of PLA_2 showing no significant increase in $sPLA_2$ expression as the parasitic load increases (Figure 5.5). This is similar to $sPLA_2$ expression pattern when flies were infected with blood stream form trypanosomes (see section 5.3.3). Thus the expression of $sPLA_2$ following trypanosome infection appears not to be dependent on the stage of the parasite.

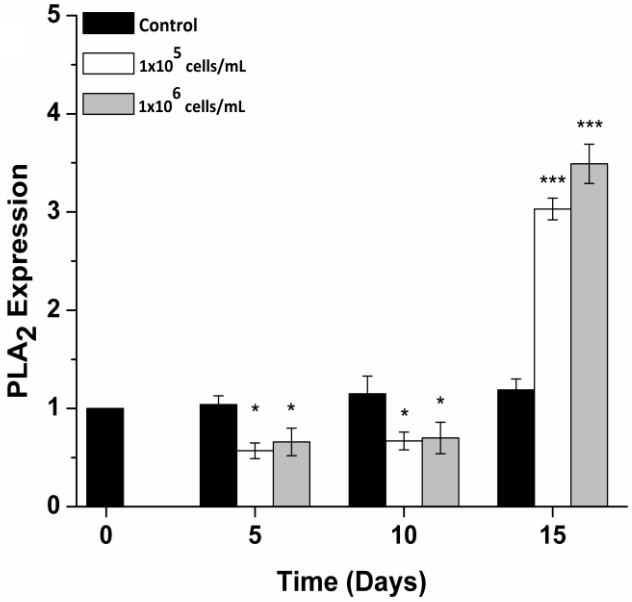

Figure 5.5 Timecourse of $sPLA_2$ expression after infection with *T. b. brucei* PCFs. Analysis of $sPLA_2$ transcript expression using qPCR. Teneral flies (n=50) were infected with *T. b. brucei* and dissected 5, 10 and 15 days post-infection. Midguts were collected and analysed for $sPLA_2$ transcript expression using qPCR. Expression levels of $sPLA_2$ were presented relative to the level in uninfected controls. The transcript levels of *G. m. morsitans* α tubulin and β tubulin were measured to normalize for differences in the concentration of cDNA templates between samples. Level of significance between test samples and control is indicated by asterisks, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data are shown as mean value ± SE (n = 3).

5.3.5 Immune stimulation of flies with Gram-positive bacteria results in a significant change in $sPLA_2$ expression

It has been demonstrated that when tsetse flies are immune challenged with bacteria before receiving a trypanosome-infected blood meal they become less susceptible to trypanosome infection (Hao et al., 2001). In as much as this phenomenon has been linked to the induction of antimicrobial peptides, there might be other factors involved (Hao et al., 2001, Hao et al., 2003). In order to check if the expression of $sPLA_2$ is specific for trypanosome infection or is it influenced by the presence of any pathogen, flies were fed with Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria. Flies were fed live *Escherichia.*

coli and live *Staphyloccocus aureus*. Flies were dissected at 5, 10 and 15 days after immune challenge to extract midgut tissues which was analysed for $sPLA_2$ transcript expression using qPCR.

The result indicates that there was a significant induction (~9-fold) of *$sPLA_2$* expression in flies challenged with live *S. aureus* compared to those receiving *E. coli* or uninfected controls (Figure 5.6). The induction in *$sPLA_2$* occurs as early as 5 dpi and remains high up to 15 dpi, where it expression was at least 2-fold higher than that detected in flies infected with *T. brucei*.

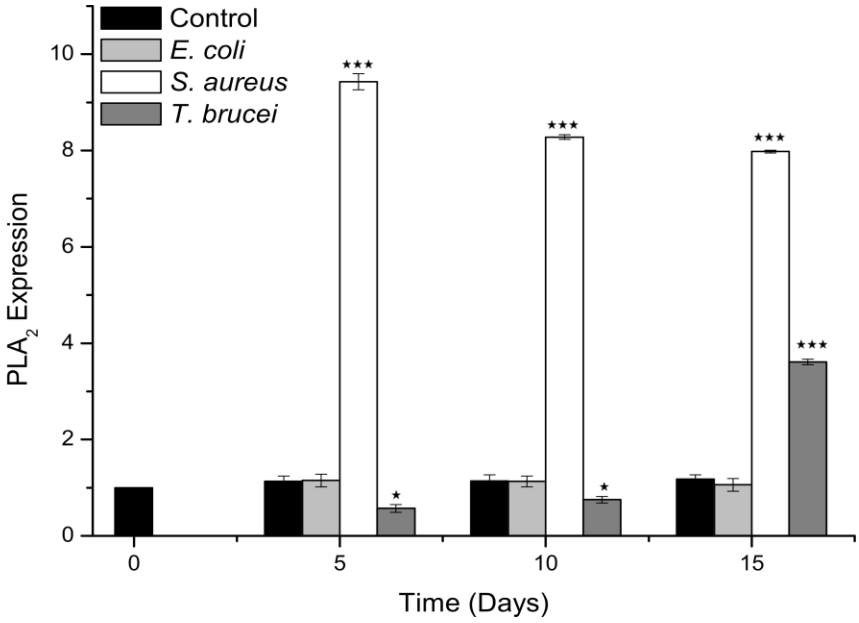

Figure 5.6 Timecourse of sPLA2 expression after immune challenge with bacteria. Analysis of $sPLA_2$ transcript expression using qPCR. Teneral flies (n=50) were fed blood meal spiked with live *S. aureus* and *E. coli* and dissected at 5, 10 and 15 days after the infectious blood meal to extract midgut tissues which were analysed to check for $sPLA_2$ transcript expression using qPCR. Challenging flies with live *S. aureus* resulted in a sharp increase in PLA_2 expression from day 5 while live *E. coli* did not result in any significant alteration in the expression of $sPLA_2$ compared to controls. Expression levels of $sPLA_2$ were presented relative to the level in uninfected controls. The transcript levels of *G. m. morsitans α-tubulin* and *β-tubulin* were measured to normalize for differences in the concentration of cDNA templates between samples. Level of significance between test samples and control is indicated by asterisks, *$p < 0.05$, ***$p < 0.001$. Data are shown as mean value ± SE (n = 3).

5.3.6 Immune stimulation of flies with dead bacteria or dead PCFs does not affect sPLA$_2$ expression

To investigate if the rise in PLA$_2$ expression can be triggered only by the pathogen molecules, a timecourse experiment in which flies were challenged with dead procyclic trypanosomes and dead bacteria was performed. Teneral flies were fed blood meals containing dead procyclic trypanosomes (1x10^5 cells/ml and 1x10^6 cells/ml) and dead *S. aureus* and dead *E. coli*. Flies were dissected at 5, 10 and 15 days after immune challenge to extract midgut tissues for sPLA$_2$ transcript expression analysis using qPCR. If the rise in sPLA$_2$ expression is as result of presence of any foreign body, then we would expect a rise in sPLA$_2$ expression after flies were challenged with dead PCFs and dead bacteria. Figure 5.7, panel A shows that feeding flies with dead procyclic trypanosomes (both 1x10^5 cells/ml and 1x10^6 cells/ml) did not result in any significant change in sPLA$_2$ expression in the midgut compared to uninfected controls. Likewise, immune challenge of flies with dead bacteria (dead *S. aureus* and dead *E. coli*) did not produce any significant change in the expression of sPLA$_2$ in the midgut compared to uninfected control (Figure 5.7, panel B). This suggests that expression of midgut *sPLA$_2$* is probably dependent on the density of live pathogens, but does not respond to the presence of dead cells or molecules derived from, although it remains to be determined whether it can be triggered by increasing the amount of surface molecules from either trypanosomes or bacteria.

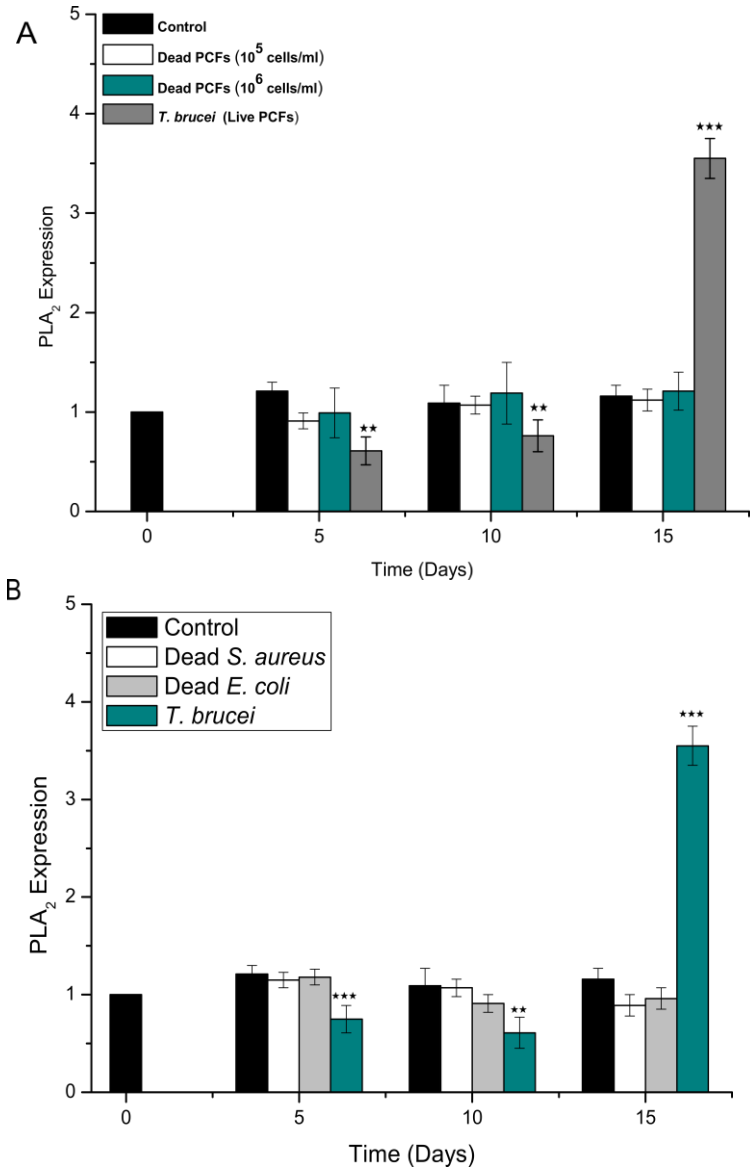

Figure 5.7 Timecourse of sPLA2 expression after immune challenge with dead trypanosomes and dead bacteria. Teneral flies were fed blood meal containing dead *T. b. brucei* (A) and dead *S. aureus* and *E. coli* (B). Flies (n = 35) were dissected at 5, 10, and 15 days post-infection to collect midgut tissues for $sPLA_2$ transcript analysis. Feeding flies with dead *T. b. brucei* PCFs did not cause any significant change in $sPLA_2$ expression compared to controls. Likewise challenging *S. aureus* or dead *E. coli* did not result in any significant change in the expression of $sPLA_2$ compared to controls. Expression levels of PLA_2 were presented relative to the level in uninfected controls. The transcript levels of *G. m. morsitans* α tubulin and β tubulin were measured to normalize for differences in the concentration of cDNA templates between samples. Level of significance between test samples and control is indicated by asterisks, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data are shown as mean value ± SE (n = 3).

5.3.7 Effect of *G. m. morsitans* s PLA_2 on trypanosome viability

To determine if $sPLA_2$ protein has trypanocidal activity, a Minimum Inhibitory Concentration (MIC) assay was performed on trypanosomes. PCF forms of *T. b. brucei* parasites were cultured with varying concentrations of recombinant *G. m. morsitans* $sPLA_2$ (rec-$sPLA_2$). Following incubations for 66-72 hours, parasite number was counted while the viability of the parasites were measured by determining their metabolic activity using alamarBlue substrate. The results are shown in Figure 5.8. The parasites were inhibited at high concentrations (50 µg/mL) of rec-$sPLA_2$ because there was a significant reduction in the number of parasites but lower concentrations of *G. m. morsitans* PLA_2 seem to discretely aid parasite growth (Figure 5.8 A). A similar result was obtained when proliferation of the parasites was measured using alamarBlue substrate. The lowest intensity of fluorescence was recorded at 50 µg/mL while at lower concentrations (0.1 – 5.0 µg/mL) the intensity of fluorescence was similar to or slightly higher than the control (0 µg/mL) (Figure 5.8 B). This shows that parasite growth is comparable to or slightly higher than the control while there was inhibition of growth at 50 µg/mL. The results indicate that *G. m. morsitans* $sPLA_2$ is trypanocidal at higher concentrations while parasite growth seem to be slightly aided at lower concentrations of *G. m. morsitans* $sPLA_2$.

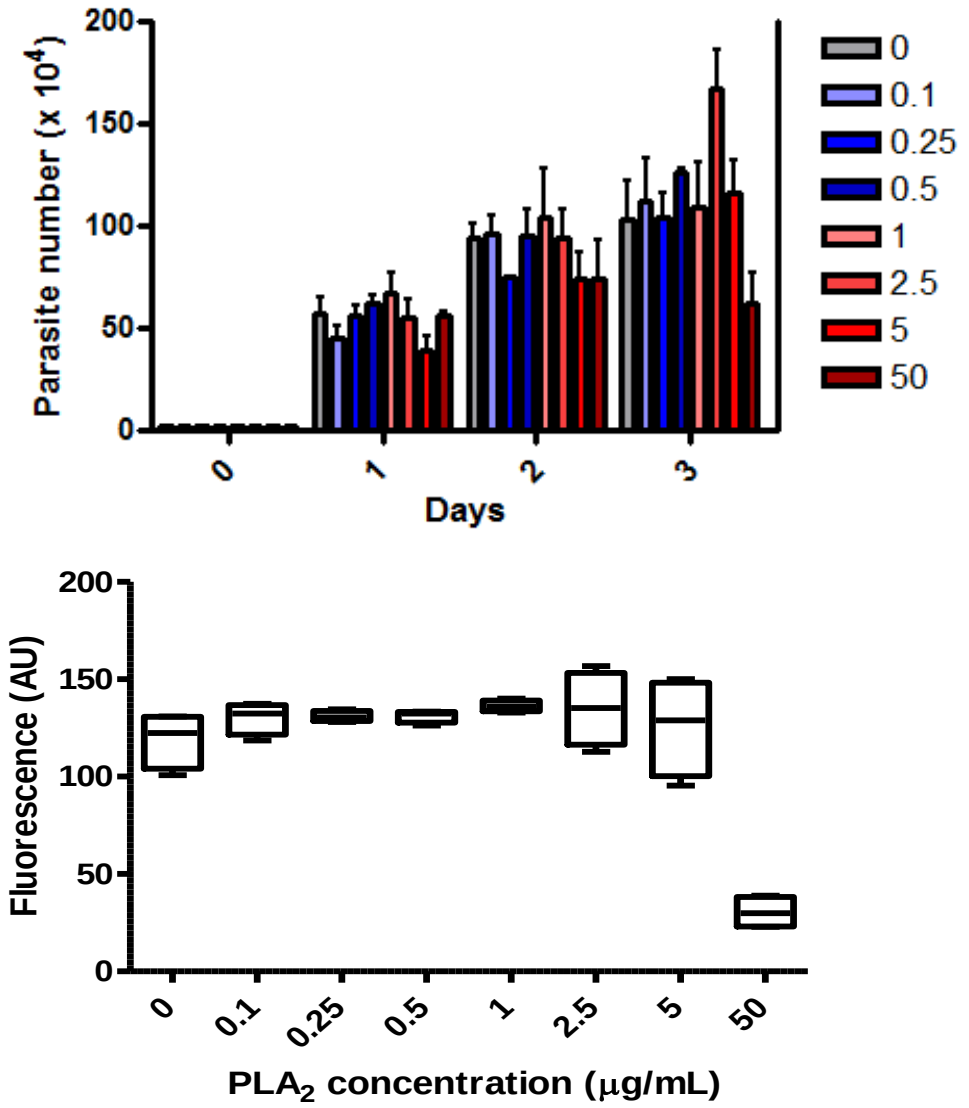

Figure 5.8 Inhibition of parasite growth by recombinant G. m. morsitans sPLA$_2$. MIC assay was performed on PCF of *T. b. brucei*. **Panel A:** Measurement of parasite growth after incubation at varying concentrations of recombinant *G. m. morsitans* PLA$_2$. Parasite numbers were counted at 0, 24, 48 and 72 hours after incubation. At 0 hours there was no increase in the number of parasites, at 24 and 48 hours there was no significant difference in the number of parasites between the various protein concentrations. After 72 hours incubation, lower concentrations of *G. m. morsitans*PLA$_2$ seem to aid parasite growth with the highest number of parasite recorded at 2.5 µg/mL while there was an inhibition of parasite growth at 50 µg/mL. **Panel B:** Detection of cell growth using alamarBlue. The alamarBlue assay was used to measure cell proliferation and the intensity of fluorescence is proportional to number of cells. The parasites were inhibited by *G. m. morsitans* sPLA$_2$ at high concentrations (50 µg/mL), while parasite growth seem to be aided at lower concentrations of *G. m. morsitans* rec-sPLA$_2$.

5.4 Discussion

Although insects lack adaptive immunity, they rely mainly on innate immunity (cellular and humoral) for defense against invading pathogens. Although the chitinous exoskeleton and the peritrophic matrix lining the midgut epithelia are formidable physical barriers to infection, many organisms succeed in breaching these barriers, but once inside the insect body, the invaders are confronted with cellular and humoral defense mechanisms. In insects humoral immunity involves the induced biosynthesis of a wide range of anti-microbial proteins, the action of lysozymes and the release of prophenoloxidase (PPO) (Stanley and Kim, 2011). Humoral immunity is linked to Toll and Immune Deficiency (Imd) pathways, which are signal transduction systems that enable the host to differentiate different classes of infecting microbes and express specific genes encoding anti-microbial peptides appropriate to the infecting microbe (Leulier et al., 2003, Hoffmann, 2003). The onset of humoral immunity is relatively slow (6-12 hours after infection) compared to cellular immune reactions (Stanley, 2006a).

It has been suggested that eicosanoids mediate cellular, as opposed to humoral, immune reactions because inhibiting eicosanoid biosynthesis in the tobacco hornworms (*Manduca sexta*) impaired the ability of the insect to clear injected bacteria within 2 – 4 hours after immune challenge (Stanley-Samuelson et al., 1991). Bacterial infection of the true armyworm, *Pseudaletia unipuncta* resulted in an increase in PLA_2 activity as well as increased biosynthesis of $PG_{2\alpha}$ 30 minutes post-infection (PI) (Jurenka et al., 1999). Also several other experiments have revealed that immune challenge of insects stimulated increased PLA_2 activity (Tunaz et al., 2003, Yajima et al., 2003, Shrestha and Kim, 2010). These experiments support the idea that PLA_2 is involved in both cellular and humoral immune reactions to infection in insects. However, since trypanosomes do not enter the haemocoel, PLA_2 may be involved in humoral immune response in tsetse. Compared to the work on Toll and other intracellular pathways, there is relatively very little knowledge on signaling mechanisms involved in mediating or coordinating cellular immune responses. Prostaglandins and other eicosanoids are involved in variuos aspects of cellular immune reactions to microbial infections (Stanley, 2000, Stanley, 2006b).

Although the role of secreted PLA_2 proteins in some insects have been investigated, there is no information about the role of $sPLA_2$ in tsetse immune response. Since $sPLA_2$ was one of the genes that were differentially expressed in the midgut of refractory flies, and based on the fact that $sPLA_2$ plays an important role in insect cellular immunity, we decided to investigate the role of this protein in the establishment of infection in tsetse following trypanosome challenge. In this chapter I investigated the functions of $sPLA_2$ in the establishment of trypanosome infection in the midgut of tsetse. RNAi ablation of of *sPLA₂* resulted in a significant increase in the susceptibility of the fies to trypanosome infection, indicating that $sPLA_2$ is involved in the fight against infection by trypanosomes. This could be as a result of the direct action of $sPLA_2$ on the parasites since $sPLA_2$ class XIIA are known to possess antimicrobial properties. However, it cannot be ruled out if $sPLA_2$ is involved in some signaling pathways, such as the eicosanoid biosynthesis pathway leading to the production of prostaglandins and other eicosanoids (which are involved in cellular immune reactions to microbial infection) (Stanley, 2000, Stanley, 2006b), although this activity appears not to be intracellular or cell-bound.

It has been demonstrated previously that PLA_2 from snake venom can prevent *P. falciparum* oocyst formation by preventing midgut attachment of ookinete thereby inhibiting the full development of malaria parasite in mosquitoes (Zieler et al., 2001). The mechanism of action of $sPLA_2$ on the parasite killing is not well understood, but it is known that $sPLA_2$ proteins have a direct (porin-like) effect on the parasite and that enzymatic activity is not a prerequisite for antiparasitic activity. It was therefore suggested that $sPLA_2$ associates with the midgut of surface of of mosquitoes thereby preventing ookinete attachment and thus preventing further development of the parasite in the midgut of the mosquito vector (Zieler et al., 2001).

To investigate if the presence of trypanosomes in the midgut of tsetse leads to a suppression of $sPLA_2$ expression, teneral flies were infected with BSFs of *T. b. brucei* and the profile of $sPLA_2$ transcript expression was analysed over a period of 14 days using qPCR. There was a significant reduction in the expression of $sPLA_2$ compared to uninfected control suggesting that trypanosomes suppress $sPLA_2$ expression upon invading tsetse. However, there was a sudden increase

in $sPLA_2$ expression on day 14 after trypanosome infection. This is an intriguing and unprecedented phenomenon. Two questions come to mind as to why the sudden increase in $sPLA_2$ expression: Firstly, does the increase in expression coincide with the transformation of the BSFs to procyclics, secondly, is the fly responding to an increase in cell density as a way of keeping the parasites at bay to prevent being overwhelmed. To address the first question I infected teneral flies with procyclics to investigate if the increase in $sPLA_2$ expression is triggered by the onset of the procyclic forms of the parasite, in which case we expect to see an increase in $sPLA_2$ expression right from the onset. This, however was not the case as the $sPLA_2$ expression profile followed a similar pattern to that obtained when flies were infected with BSFs.

To address the second question I infected flies with two different parasite loads (1×10^5 cells/ml and 1×10^6 cells/ml) to see if there will be a difference in $sPLA_2$ expression between the two treatment groups. Again the expression profile for $sPLA_2$ was similar to that obtained when flies were infected with either BSFs of PCFs, however, $sPLA_2$ expression was higher on day 14 in flies infected with 1×10^6 parasites/ml compared to flies receiving $1x10^5$ parasites/ml in their blood meal. This suggests that more $sPLA_2$ is expressed as the level parasite infection increases, which is in aggrement with the lack of induction when challenged with dead trypanosomes and bacteria. In order words, the flies expressed more $sPLA_2$ as the parasites proliferate and actively increase in number in the midgut. This could be a way of limiting the number of parasites in the midgut and preventing them from overwhelming the flies, although the expression may not be enough to fully erradicate the infection.

It has been shown that some bacteria belonging to the Enterobacteriaceae family (Park et al., 2004, Park and Stanley, 2006, Kim et al., 2005, Park et al., 2003, Park and Kim, 2000) and some protozoan parasites (Garcia et al., 2004) suppress the immune defences of their insect hosts by inhibiting eicosanoid biosynthesis. Bee venom PLA_2 has been shown to have antimicrobial activity on some Gram-negative bacteria (Tatu, 1989). $sPLA_2$ from mouse has been reported to exhibit a bactricidal action on both *E. coli* and *Salmonella spp*. (Koduri et al., 2002, Harwig et al., 1995). Also human $sPLA_2$ and group III PLA_2 possessed antibacterial activity against Gram-positive and Gram-negative

bacteria respectively (Qu and Lehrer, 1998, Koduri et al., 2002). However, there are relatively few reports describing the effects of PLA_2 on parasites. It has been shown that *Trypanosoma rangeli*, a protozoan parasite of *Rhodnius prolixus* inhibits the release of arachidonic acid for eicosanoid biosynthesis(Garcia et al., 2004).

The activity of the recombinant $sPLA_2$ was measured fluorimetrically to determine if it is enzymatically active or not. The rec-$sPLA_2$ was found to be enzymatically active and the enzyme activity increased with substrate concentration (Appendix 2). To determine if $sPLA_2$ is trypanocidal, we used rec-$sPLA_2$ to perform a Minimum Inhibitory Concentration (MIC) assay on trypanosomes to ascertain the effects $sPLA_2$ on the survival of *T. b. brucei*. PLA_2s are known to possess antimicrobial acitivity. From our data, it appears that the presence of $sPLA_2$ at low concentration does not seem to inhibit the growth of trypanosomes, while at higher concentration there was an inhibition of trypanosome growth suggesting that $sPLA_2$ is trypanocidal at high concentrations. In as much as *in vitro* assays are important to investigate and identify cellular/molecular working mechanisms, they are performed with cells or biological molecules outside their normal biological environment and therefore cannot easily mimic *in vivo* exposures. The concentration of PLA_2 needed to inhibit trypanosome growth *in vivo* might not be the same with the concentration of PLA_2 *in vivo*.

This is in line with what has been observed using bee venom PLA_2 ($bvPLA_2$) which has been demonstrated to kill trypanosomes at high concentrations (Boutrin et al., 2008). At lower concentrations of $bvPLA_2$, the parasites are not completely killed, in fact, growth comparable to or higher than the controls was observed (Boutrin et al., 2008). It is thought that the PLA_2 acts by disrupting the membranes of the parasites (Boutrin et al., 2008). Trypanosomes expresses a membrane bound PLA_2 activity, which is known to be involved in the regulation of calcium metabolism (Eintracht et al., 1998, Catisti et al., 2000). The activity of PLA_2 in trypanosomes produces arachidonic acid (AA) which in turn activates calcium influx, thereby serving as a positive feedback regulator to PLA_2 (Eintracht et al., 1998). If membrane disruption occurs, calcium regulation in trypanosomes could be lost because there will be uncontrolled efflux of calcium

brought about by holes in the membrane caused by PLA_2 digestion (Boutrin et al., 2008). Also, higher levels of intracellular AA may be produced as a result of the PLA_2 reaching the cytoplasm and this may lead to accumulation of higher concentrations of intracellular Ca^{2+} which in combination with reactive oxygen species could trigger calcium-dependent fragmentation of nuclear DNA, loss of motility and ultimate death of trypanosome cell death (Parsons and Ruben, 2000, Ridgley et al., 1999). It means therefore that with or without significant membrane damage, $sPLA_2$ may still be able to disrupt the regulation of calcium leading to death. This is in contrast with what has been observed in mosquitoes in which snake venom PLA_2 prevented oocyst formation but does not have a direct effect on the plasmodium parasite (Zieler et al., 2001). It remains to be determined how the *G. morsitans* sPLA2 kill trypanosomes, but the protein may act either on the parasite surface forming pores or intracellularly (e.g. lysosome), in which case it may altere parasite homeostasis.

The downregulation of $sPLA_2$ early on during the infection process was not surprising since the parasites will try to suppress the expression of $sPLA_2$ (and other immune-related molecules) to establish an infection. The fly on its part starts to express more $sPLA_2$ as the infection progresses to eliminate the proliferating parasites. Although the expression of $sPLA_2$ was found to be down-regulated in refractory flies, this could have been influenced by the timing of midgut dissection following the infectious blood meal (3 dpi). This was done to maximise the opportunity of finding the genes involved in the elimination of the invading parasites, since this period is characterised by a process of attrition that leads to complete elimination of parasites in some flies (Gibson and Bailey, 2003).

Based on our findings we propose the following: (i) tsetse $sPLA_2$ is involved in immune response during trypanosome infection since its downregulation partially reverses the refractory phenotype; (ii) following trypanosome infection, the expression of $sPLA_2$ is suppressed by the parasites in order to create a conducive environment in which to proliferate and (iii) expression of $sPLA_2$ may be linked to the tsetse Toll pathway as a challenge with *S. aureus*, but not *E. coli* induces a massive expression of this protein. In response to the increase in the number of parasites, the fly tries to fight back and starts to express more $sPLA_2$

in a bid to possibly eliminate the parasites or reduce the number of parasites to a level that will not overwhelm the fly and result in a fitness cost (Figure 5.8).

Attention is turning towards blocking the transmission cycle in the tsetse fly host, but before this will become a reality the it is absolutely important to understand the molecular mechanisms underlying the complex interactions between trypanosomes and their tsetse fly vector. Interestingly tsetse flies are naturally refractory (resistant) to trypanosome infection and the genes involved in refractoriness can be used to fight the parasite in the fly by constitutively expressing these genes in the midgut of the fly in close proximity to the invading parasites. Therefore, it is necessary to better understand how tsetse $sPLA_2$ works as it can be used as a paratrangenesis control tool. Since tsetse flies reproduce by adenotrophic viviparity this can only be done paratransgenetically by using one of the endosymbionts found in the midgut (e.g. *Sodalis glossinidus*) to express the genes in the midgut. The midgut of tsetse, however, is not a particulary friendly environment, being filled with degradative enzymes that are also employed in the fight against invading parasites.

Candidate genes must therefore be able to withstand the harsh midgut environment. One gene that fits this bill is the PLA_2. PLA_2 possesses some unique properties that make them very suitable for expression in the degradative environment of tsetse and one of these properties is that it is exceptionally stable and can withstand the degradative environment of the tsetse midgut lumen (Zieler et al., 2001). Furthemore, $sPLA_2$ is also expressed in tsetse saliva, which raises the fascinating question as to whether this enzyme helps to control colonisation of the fly salivary glands by the parasite epimastigotes. In summary, I have demonstrated that a reduction in the expression of PLA_2 in tsetse made the flies more susceptible to trypanosome infection suggesting that $sPLA_2$ is actively involved in immune response to trypanosome infection. Furthermore, I have shown that the invading parasites suppress the expression of PLA_2 early on during the infection process to create a favourable environment for their proliferation. The data presented here will be helpful in understanding the mechanisms involved in tsetse-trypanosome interaction.

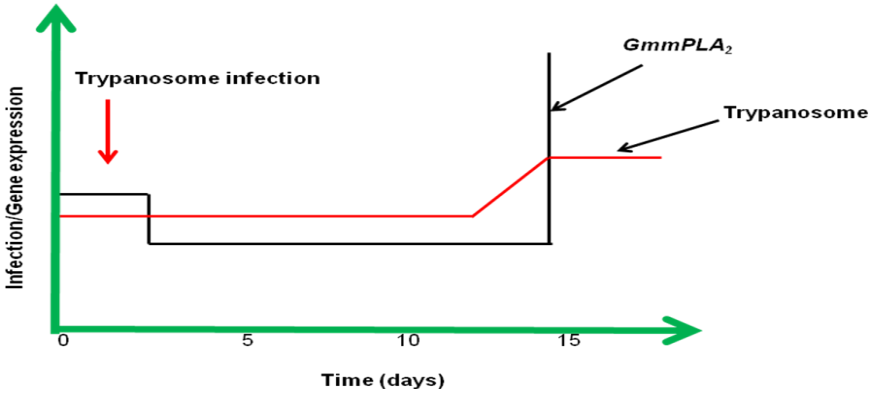

Figure 5.9 Schematic representation of $sPLA_2$ expression during the cross-talk between tsetse and trypanosomes. Trypanosomes invade tsetse midgut and secrete or express substances aimed at suppressing $sPLA_2$ expression thereby crippling tsetse immune response. This creates a favourable environment for the parasites to flourish. In response to the increase in parasitaemia, the flies fight back by increasing the expression of $sPLA_2$ to possibly eliminate or at least prevent the parasites from overwhelming the flies.

Chapter 6
General discussions and conclusions

The main objective of the work presented in this thesis was the identification and characterisation of some genes that are differentially expressed in the midgut of tsetse flies that are refractory to trypanosome infection. By doing this I intended to shed more light on the molecular mechanisms underlying the interaction between trypanosomes and tsetse which is not fully understood. RNAi was used to knockdown the genes of interest (i.e. *Chit, SPI, OGT* and *sPLA$_2$*) and then observed their effects on the susceptibility of the flies to trypanosome infection. In this chapter, a summary of the main findings with regards to the research questions are presented together with general conclusions arising from the findings outlined in this study.

African trypanosomes undergo an obligatory and complex developmental cycle in tsetse flies before maturing into mammalian infective forms and therefore rely on them for transmission and dissemination. To better understand the transmission dynamics of these parasites therefore, it is imperative that the complex interaction between the parasite and the tsetse fly is understood. Against this backdrop, coupled with the difficulty in developing a vaccine for the disease because of the phenomenon of antigenic variation exhibited by the parasites, there has been a shift in paradigm towards blocking the transmission of the parasite by disrupting its developmental cycle in the vector using paratransgenetic approach.

The identification and characterisation of genes that have the potential of disrupting the life cycle of the parasites when expressed in the vector is an important step in paratransgenetic technology. To this end, there have been reports of the development of transmission-blocking antibodies that have the ability to block the development of trypanosomes in the fly (Nantulya et al., 1987, Nantulya and Moloo, 1988). An alternative approach is the use of genes that are employed by the insects naturally to fight infection as potential effectors. Insects generally are known to mount robust innate immune responses in reaction to the

presence of pathogens and in fact, can clear these pathogens. These immunity genes are therefore potential candidates that can be identified and characterised to be used as effector molecules. Tsetse flies are known to be naturally refractory (resistant) to trypanosome infection because of their innate ability to mount robust immune responses to invading trypanosomes clearing the parasites in most cases resulting in a few flies being able to nurture and transmit the parasites to mammalian hosts (Gibson and Bailey, 2003). As a result of this, it has been speculated that either the immune responses are belated or are not expressed close enough to the parasites, or both, and as a result are not able to combat the parasites. Constitutive expression of these antitrypanosomal molecules in the midgut of tsetse using one of its endosymbionts therefore looks promising as a means of enhancing the immune responses of the fly. This method has been successfully used to reduce the development of *Trypanosoma cruzi* infections in the hindgut of triatomine vectors of Chagas disease (Durvasula et al., 1997, Beard et al., 1998). Also the use of paratransgenetic approach to control parasite transmission has been applied to other vector-borne diseases such as sand fly-transmitted leishmaniasis (Hillesland et al., 2008). To use this approach for African trypanosomiasis we must first of all understand the molecular mechanisms involved in the crosstalk between tsetse and trypanosome. Major findings in this thesis are summarised below.

- We identified a set of differentially expressed genes in the midgut of self-cleared trypanosome infected flies. Bioinformatic analysis revealed a total of 17 genes that were up-regulated and 38 genes that were down-regulated in the midguts of flies that had self-cleared trypanosome infections compared to non-trypanosome-challenged tsetses. It has been observed that only a small proportion of genes are upregulated in tsetse in response to trypanosome or bacterial challenge while the majority of genes (including immune related genes) are downregulated (Lehane et al., 2003a).

- In this study, I have shown that *Chit, OGT, SPI* and *sPLA$_2$* are involved in the immune responses of the tsetse fly during trypanosome infection,

although only *OGT* gene was found to be up-regulated in refractory flies. It has been shown that trypanosome or bacterial challenge of flies leads to a significant increase in the number of down-regulated genes (Lehane et al., 2003a). The knockdown of each of the candidate genes led to a significant increase in midgut infections. In arthropods the immune system can be said to be highly dependent on the regulation of chitin synthesis and degradation since most of the features of the immune system such as the phenoloxidase (PO) cascade, melanization and antimicrobial peptides are linked to chitin metabolism (Beckerman et al., 2013). Also PO cascade is thought to be triggered by chitin-binding antimicrobial peptides (Nagai et al., 2001) and melanin a major feature of invertebrate immune system is thought to be regulated by chitin metabolism (Marmaras et al., 1996, Walker et al., 2010).

Chitinase which is directly involved in chitin degradation and modification (Muthukrishnan et al., 2012), can be said to play an important role in insect immune response. The inhibition of chitinase can also have a negative impact on the defensive roles of the peritrophic matrix since chitinase is involved the regulation of chitin (a major component of the peritrophic matrix) synthesis and degradation thereby compromising the defensive role of the peritrophic membrane. It is therefore expected that RNAi-mediated knockdown of chitinase will make the flies more susceptible to trypanosome infection. Accordingly, there was a significant increase in midgut trypanosome infection prevalence in dsRNA-CHT knockdown flies compared to controls.

- I was also able to demonstrate that OGT is involved in immune response when flies are challenged with trypanosomes. The use of OGT as an immune modulator is thought to be evolutionarily conserved in both vertebrates and invertebrates (Bond et al., 2014). OGT catalyses the transfer of *O*-linked GlcNAc residues to both nuclear and cytosolic proteins, a post-translational modification (PTM) that plays a vital role in numerous cellular signalling pathways involving growth, metabolism, cellular stress and host-pathogen interactions (Hanover et al., 2010).

There is the notion that inappropriate O-GlcNAcylation of specific, key OGT targets brought about by the absence of OGT will lead to immunodeficiency (Bond et al., 2014). Consistent with this notion, a significant increase in midgut infection was observed in flies injected with dsRNA-OGT compared with uninjected and dsRNA-GFP-injected controls. It has been shown that the prevention of O-GlcNAcylation of proteins leads to significant loss of nitric oxide (NO) and cytokine production (Ryu and Do, 2011) which are known to play important roles in early host defence against invading pathogens (Janeway and Medzhitov, 2002, Beutler and Rietschel, 2003).

This thesis provides strong evidence that OGT is deployed during trypanosome infection to increase O-GlcNAcylation of proteins. It is our view that the knockdown of OGT led to a reduction in O-GlcNAcylation of proteins which results in significant reduction in NO production thereby inhibiting the ability of the flies to fight the invading parasites. This therefore results in increased susceptibility of the flies to trypanosome infection. It has been suggested that a complex interaction exists between NO signaling and O-GlcNAcylation pathway (Baudoin and Issad, 2014). Innate immune signaling is known to be initiated by specific interaction of pathogen ligands with Toll-like receptors (TLRs), which induces iNOS expression, and, subsequently, the production of NO, which acts as both bactericidal agent as well as an intracellular mediator (Baudoin and Issad, 2014). The knockdown of OGT is known to abolish NO-induced effects in mice (Liu et al., 2014). Therefore, it is possible that tsetse OGT controls immune-related pathways like production of NO, which is known to be deleterious for trypanosomes in the fly.

- An important component of the humoral immune response in arthropods is the prophenoloxidase-activating system (proPO-AS) (Cerenius and Soderhall, 2004, Kanost et al., 2004). It is induced in response to pathogen invasion with limited activation of the prophenoloxidase (proPO) precursor into the active phenoloxidase (PO), culminating in the production of melanin and associated toxic intermediary compounds

which kill invading pathogens (Franssens et al., 2008). If the active proteases involved in proPO activation are not inhibited, they can cause damage by cleaving other host proteins or trigger premature or excessive activation of proPO which may act beyond the intended target.

Serine proteinase inhibitors are an important part of controlling the responses that rely on serine proteases (such as melanisation and activation of the Toll pathway) to generate an effector response (Ragan, 2008). They are known to inhibit serine proteases involved in the Toll pathway (Levashina et al., 1999) as well as the melanisation pathway in a host of insects such as *D. melanogaster* (De Gregorio et al., 2002), *M. sexta* (Jiang and Kanost, 1997, Tong et al., 2005, Tong and Kanost, 2005, Wang and Jiang, 2004, Zhu et al., 2003) and *A. gambiae* (Michel et al., 2006).

Serine proteinase inhibitors therefore can be said to form an integral part of the immune response in insects and its inhibition could lead to uncontrolled action of serine proteases which apart from causing damage as a result of the cleavage of host proteins, can also result in uncontrolled or untimely activation of the proPO leading to the accumulation of toxins. The above factors could have contributed to the increased susceptibility of dsRNA-SPI knockdown flies to trypanosome infection and with this result, we have demonstrated that SPI plays an active role in tsetse immune response. This SPI is different to the series of serine protease inhibitors secreted in the tsetse midgut and with proven anti-complement activity (Ooi et al., 2015).

- As part of the investigation to understand the interaction between trypanosomes and tsetse fly, the role of PLA_2 in the establishment of infection in the midgut of *G. m. morsitans* was carried out. Chapter 5 discusses most aspects of the potential role if this enzyme in the innate immune pathway of tsetse flies.

  One aspect that remains unresolved is whether $sPLA_2$ exhibit trypanocidal activity in the fly or does it act through the eicosanoid biosynthetic pathway to release prostaglandins and other eicosanoids that are involved

in immune response? The role of PLA_2 on eicosanoid biosynthesis can be tested by inhibiting the activity of PLA_2 using a specific PLA_2 inhibitor such as dexamethasone and observing the effect on the immune response of tsetse. The addition of arachidonic acid will restore the production of eicosanoids and boosting of immune reactions (Figure 6.1). In addition, the mode of action of tsetse $sPLA_2$ on trypanosomes is not known although it may be acting by disrupting the trypanosome membrane leading to an uncontrolled efflux of calcium resulting in cell death (Boutrin et al., 2008). Collectively, my data shows that PLA_2 plays an important role in the immune response of tsetse to trypanosome infection and we propose a model that trypanosomes inhibit $sPLA_2$ expression on invading the flies but the fly fights back as the parasites proliferate in the midgut by expressing more PLA_2 to get rid of the parasites. This is depicted in Figure 5.9.

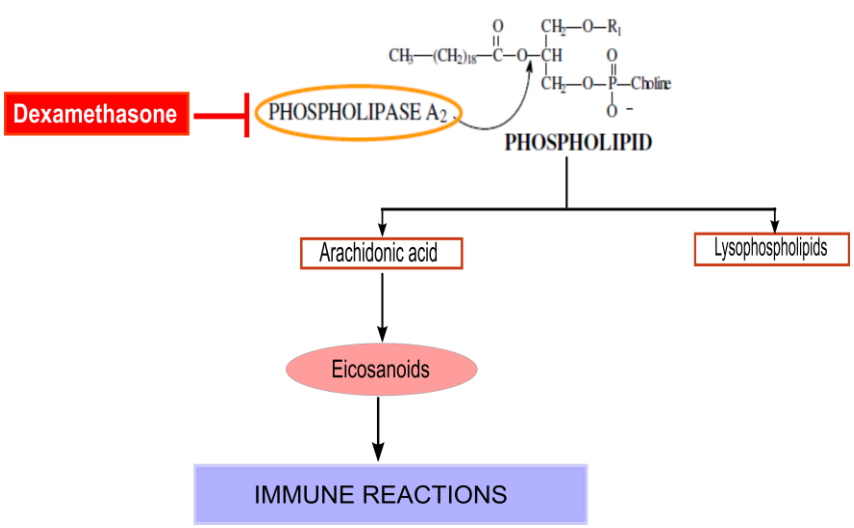

Figure 6.1 Role of PLA_2 on eicosanoid biosynthesis. Phospholipids are hydrolyzed by PLA_2 with the production arachidonic acid and the subsequent production of prostaglandins and other eicosanoids using arachidonic as substrate. In the presence of dexamethasone, a specific PLA_2 inhibitor, the immune responses are suppressed as a result of the inhibition of PLA_2 activity leading to the suppression of eicosanoid production, while the exogenous addition of arachidonic acid restores the production of eicosanoids and enhancement of immune responses

Last, there were some constrains and limitations that were encountered during the course of this study.

- The transcriptome data consists of reads from control (uninfected) and flies fed an infective bloodmeal (containing a mixture of self-cured (refractory) and infected (susceptible) flies). Comparison of gene expression was therefore between uninfected flies and flies challenged with trypanosomes. The group of flies challenged with trypanosomes will therefore consist of refractory and susceptible flies. There was no opportunity to compare gene expression between refractory (self cured) and susceptible flies.

- Although qPCR was used to check for knockdown and transcript expression, western blot analysis was only used to corroborate expression of $sPLA_2$ due to non availability of suitable antibodies for the other proteins and time constraints.
- Also, it would have been interesting to knockdown *O*-GlcNAcase (OGA; the enzyme that removes OGT from proteins) and see what effect it will have on the expression of OGT and trypanosome infection prevalence.
- Although dsRNA was delivered by injection, it will be nice to feed the dsRNA to see if the same level of knockdown and effect on trypanosome establishment will be achieved. Moreover this will avoid pre-stimulating the immne system of the fly by injection.
- For $sPLA_2$, because of time constraints, we could not proceed with the knockdown of some genes involved in the Toll/Imd pathways to observe what effect it will have on the expression of $sPLA_2$ since it has been observed that the activation of $sPLA_2$ is functionally linked to Toll/Imd signal pathways (Shrestha and Kim, 2010).

Future work

- It will also be interesting to feed tsetse with *Sodalis glossinidius* and then measure $sPLA_2$ expression. This will be an interesting experiment since *S. glossinidius* has been linked with increased susceptibility to trypanosome infection in tsetse (Welburn and Maudlin, 1999) based on its

chitinolytic activity which produces lectin (Welburn et al., 1993). However its effect on $sPLA_2$ expression has never been investigated.

- Also since *S. glossinidius* belongs to the Enterobacteriaceae family some of which have been implicated in the impairment of insect host immunity by inhibition of $sPLA_2$s (Park and Kim, 2000, Park et al., 2003, Park et al., 2004, Park and Stanley, 2006, Kim et al., 2005), it will be interesting to see if it does inhibit $sPLA_2$ in tsetse also.
- It will be good to determine the potential link between Toll/Imd pathways and PLA_2 expression by knocking down genes (one in each pathway) and then analyse the expression of PLA_2 following challenge with *T. b. brucei*.
- Extend timecourse experiments beyond day 15 to see if PLA_2 expression is sustained beyond that time point.
- To ascertain if systemic knockdown of PLA_2 was achieved following after injection dsRNA by examining PLA_2 expression in the salivary glands using western blot.
- Analysis of the SG and PV to look at the parasite population/developmental stages and possible link between the negotiating the PV or colonization of the SG and PLA_2 expression.

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Appendix 1

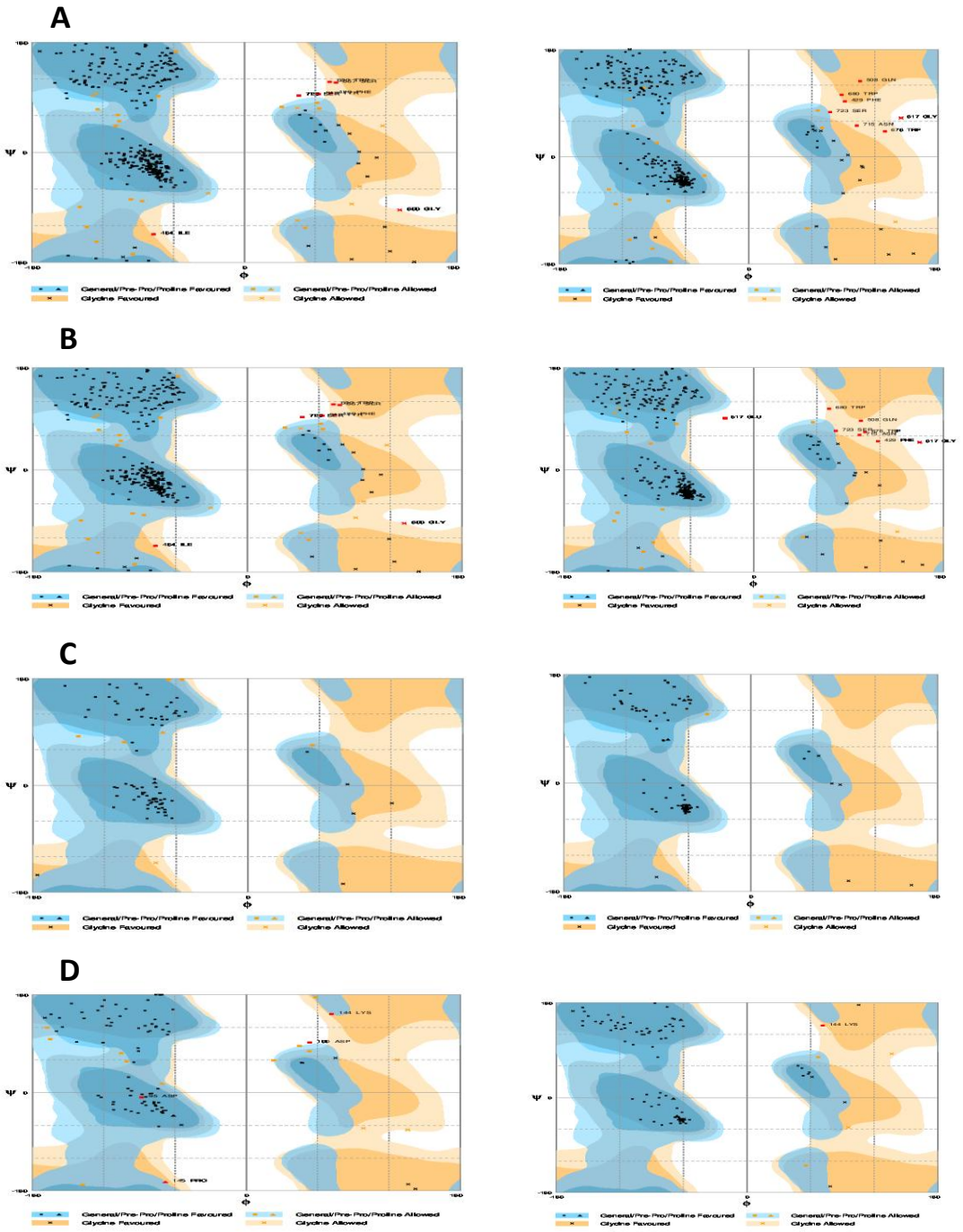

Figure A.1. Ramachandran plots of initial and final models of (A) *GmmCHT*, (B) *GmmOGT*, (C) *GmmPLA$_2$* and (D) *GmmSPI*. Number of residues in favoured region: (A) initial, 319 (91.1%), final, 327 (93.4%); (B) initial, 319 (91.1%), final, 327 (93.4%); (C) initial, 90 (92.8%), final, 96 (99.0%); (D) initial, 82 (82.8%), final, 94(94.9%). Number of residues in allowed region: (A) initial, 24 (6.9%), final, 16 (4.6%); (B) initial, 24 (6.9%), final 15 (4.3%); (C) initial, 7 (7.2%), final 1 (1.0%); (D) initial, 13 (13.1%), final, 4 (4.0%). Number of residues in outlier region: (A) initial, 7 (2.0%), final 7 (2.0%); (B) initial, 7 (2.0%), final, 8 (2.3%); (C) initial, 0 (0.0%), final, 0 (0.0%); (D) initial, 4 (4.0%), final, 1 (1.0%)

Appendix 2

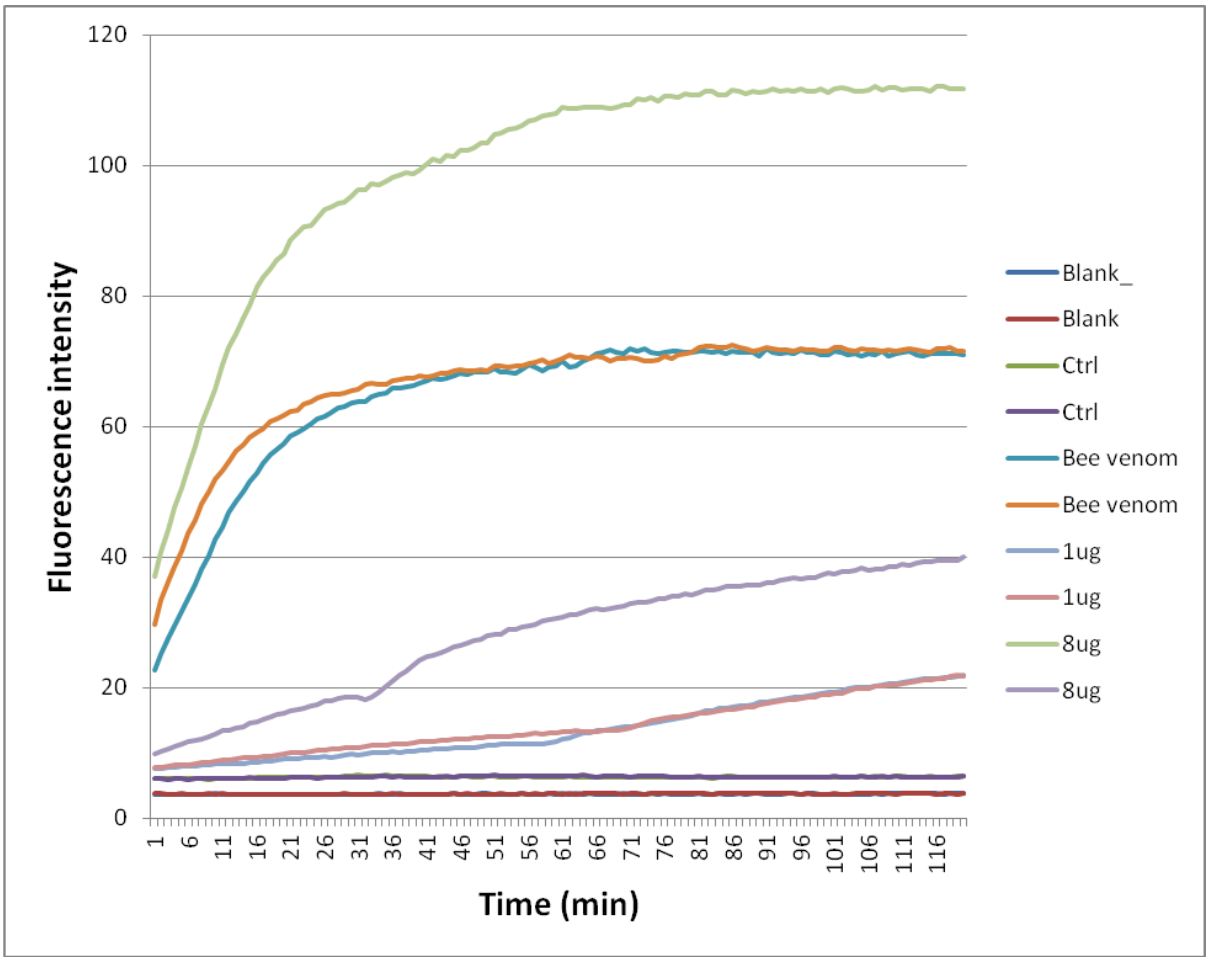

Figure A2 sPLA$_2$ activity assay. Recombinant sPLA$_2$ is enzymatically active and activity increases with protein concentration.